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STUDIES IN THE PHYSIOLOGY OF COMMENSALISM. 1. THE POLYNOID GENUS ARCTONOË

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Significant work on the role of specific chemical factors in controlling host-parasite or host-commensal relationships has been published by Thorpe and Jones (1937) and by Welsh (1930, 1931). The former authors have demonstrated the significance of olfactory conditioning in host-selection by parasitic insects. Welsh has shown that the normally positively phototactic sign of the mite *Unionicola* is reversed by a substance released from its host, the fresh-water clam *Anodonta,* and also that the sign of three species of *Unionicola* occurring in clam hosts of different genera can be reversed by substances from their hosts alone. To the writer's knowledge, however, no experimental work has been done on any of the legion of host-parasite or host-commensal associations to be found in the sea, nor has it been demonstrated whether or not in any of these a chemical bond exists between host and partner.

The members of the polynoid genus *Arctonoë* present an interesting commensal complex. These worms can readily be collected in Puget Sound both in the intertidal zone and by dredging; along with their hosts they are in many ways admirably suited to experimental work. In Table I the host genera of the three described species of *Arctonoë* are listed, as given by Pettibone (1947) and the Berkeleys (1948). It is to be noted that *A. fragilis* (Baird) is listed as commensal with certain starfish only, while *A. pulchra* (Johnson) and *A. vittata* (Grube) are commensal with certain starfish, holothurians, gastropods, chitons and terebellids. The records would seem to show that some hosts (see arrows) may harbor two species of commensals, though there are no records at hand of an individual host with more than one species of commensal. The cucumber *Stichopus,* although not listed as a host of *vittata* by these authors, is included on the basis of commensals identified for the writer by Dr. Olga Hartman, according to whom the commensals of *Stichopus* may show some characteristics of *pulchra* and some of *vittata.*

While some commensal populations of *Arctonoë* appear to be relatively distinct on anatomical grounds (markings, condition of neuropodial setae and margins of elytra), others are variable and appear to include intergrades. By dealing with the complex as made up of populations on certain specific hosts it seemed that it might be possible to cast some light on the basic physiology of the partnerships and thereby, incidently, on the taxonomic problems involved. The primary question arose: "How are these worms bound to specific hosts, existing as they may in environments

TABLE I

Hosts of the Genus Arctonoë

	fragilis (Baird)		*pulchra* (Johnson)		*vittata* (Grube)
Asteroidea	Evasterias		Solaster	←→	Solaster
	Luidia	←→	Luidia		Dermasterias
	Stylasterias		Pteraster	←→	Pteraster
	Orthasterias				Henricia
	Leptasterias				Crossaster
	Pisaster*				
Holothuroidea			Stichopus	←→	(Stichopus)
Gastropoda			Megathura*		Diadora
					Puncturella
					Acmaea
					Cidarina
					Argobuccinium
Amphineura			Cryptochiton*	←→	Cryptochiton
Polychaeta			Loimia*		Neoamphitrite

* Not recorded as hosts in Puget Sound.

where other species related to their hosts but without commensals are in close association or in actual contact with them?"

To the end of determining whether some specific chemical bond exists between commensal and host, studies were conducted at the Oceanographic Laboratories of the University of Washington at Friday Harbor, under a grant from the American Academy of Arts and Sciences. The writer wishes to express his appreciation to the Academy and to thank the Director of the Laboratories, the members of the staff and in particular Dr. Robert Hungate of Washington State College for their generosity and kind assistance.

MATERIAL

Worms of the beautiful form *A. fragilis* (Baird), commensal on the locally abundant inter-tidal star *Evasterias troschelii* Stimpson, were selected as the first animals to be used in these experiments and proved a happy choice for a number of reasons. *Evasterias* can be found in large numbers at certain points in Puget Sound where strong tidal currents prevent the deposition of sand or mud or the growth of extensive beds of algae. Stars were collected on a point of Orcas Island on Wasp Passage and on the pilings of the ferry landing at the village of Orcas. Although *Evasterias* has a more local distribution than the ever-present *Pisaster ochraceus,* when concentrations of the animals are found they are often in the same zone and in close association with *Pisaster, Pycnopodia, Solaster, Henricia, Leptasterias, Dermasterias* and other genera. Approximately thirty per cent of the *Evasterias* appeared to have commensal *Arctonoë.* The original observation of these commensals on hosts in close association with other genera of stars which are not regularly their hosts initiated these experiments.

Large numbers of the mud-star, *Luidia foliolata* (Grube), were collected by dredging in West Sound on the south side of Orcas Island. A high percentage of these stars had commensal *Arctonoë* (*pulchra-fragilis*?). This partnership did

not, however, prove as satisfactory for experimental work as *Evasterias* and its commensal, because of the tendency of the mud-stars to autotomize their arms in the live-boxes and die.

Stichopus californicus (Stimpson) with attendant *Arctonoë* (*pulchra-vittata?*) could be collected by dredging or with a long rake almost anywhere in the Sound where there was soft bottom.

Although the large numbers of the form *A. vittata* commensal with the keyhole limpet *Diadora* made this association readily available, the extreme inactivity of these worms was such as to make their use in the experimental apparatus impossible.

It was found that host *Evasterias* and *Stichopus* could be kept in live-boxes for weeks without any apparent effect on the health or activity of their commensals. The worms were found to be extremely hardy and easy to handle. The name *fragilis* is certainly a misnomer and quite clearly the result of the observations of a worker who noticed that the worms break into fractions when placed in formalin or alcohol. In the course of the experiments worms were pulled from the host with forceps, manipulated with camel's-hair brushes, placed in the test apparatus, removed and replaced on their hosts many times without any apparent effect on their health and activity other than the occasional loss of an elytron. Twenty *Evasterias* with commensals were collected at the first of the summer and kept in live-boxes for two months, and in the tests to be described with *A. fragilis,* their worms were used exclusively. The hosts were fed bivalves of various species and when a star was feeding, its commensal could often be observed lying in the fold between the arms of the star with its head directed toward the everted stomach of the star, apparently stealing a meal.

Methods

The essentially simple unit diagrammed in Figure 1 was designed to make commensals choose between two streams of water. It consisted of a glass Y-tube with a seven-and-a-half-inch stem of three-quarter-inch pyrex and two four-inch arms of one-half-inch pyrex at approximately right angles to each other. These arms were extended and bent inward by joining to them three-inch lengths of half-inch pyrex by means of rubber sleeves. This was done in order to eliminate turbulence and to give a smooth flow at the point-of-choice (B). At the open base of the Y-tube (A) was a stop-cock set in a rubber stopper, which could be removed from the Y to allow introduction of animals.

Two identical wooden aquaria with an internal dimension of $10'' \times 10'' \times 10''$ were each connected to both arms of the Y, making it possible for alternate arms of the Y to be used as test arms. Vents at the bottoms of the aquaria were connected to the extended arms of the Y by $\frac{3}{16}''$ rubber tubing. By closing a pinch clamp on any one of the four rubber connections, flow in that part of the system could be completely stopped.

Commensals to be tested were separated from their hosts for twelve hours or more prior to an experiment and kept singly in dishes with circulating sea-water, on the theory that by such a procedure the threshold of the worms to "host-factor" might be lowered. Echinoderm hosts were placed in non-circulating, though aerated, sea-water in a glass aquarium and this, in turn, was kept partially immersed in a larger aquarium of circulating sea-water to maintain a low temperature (approxi-

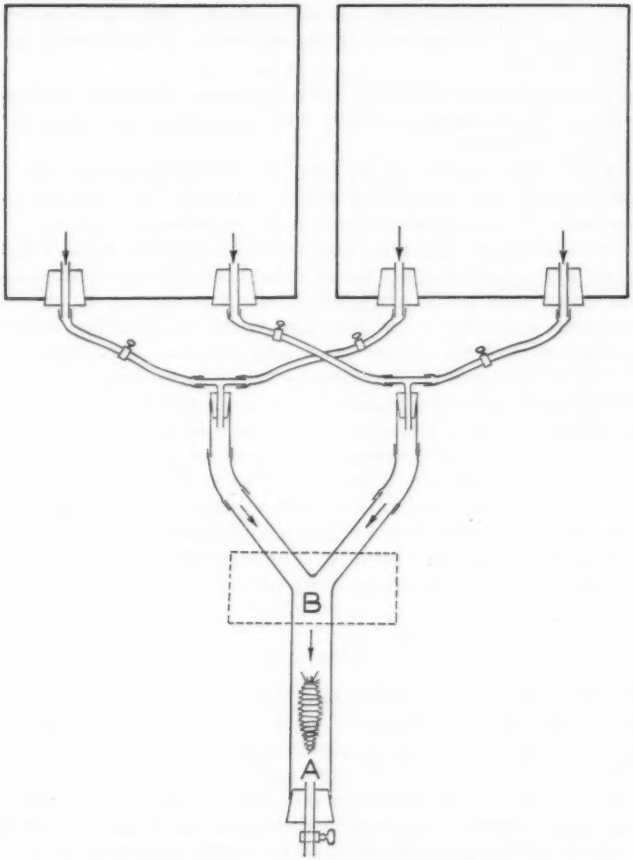

FIGURE 1. Plan of experimental apparatus.

mately 13.5° C.) over night. This was done in the hope that "host-factor" might accumulate in the water to be tested. An identical glass aquarium containing sea-water alone (or non-host to be tested against host) was treated in a similar manner. Immediately prior to a series of experiments the entire contents of the two glass aquaria including the echinoderms were transferred to the two wooden aquaria of the test apparatus. Each pinch clamp was opened to remove air from the system, both arms of the Y were flushed out from the sea-water or non-host aquarium, and the apparatus was ready for a run.

When a run was to be made, the stopper at A was removed with the four pinch clamps closed, and a worm introduced. The stopper was then replaced and the contained stop-cock opened to allow a slow but constant flow. Two of the pinch clamps were then opened at will to allow water from each aquarium to meet at the

point-of-choice (B). When the worm had moved up to the point-of-choice and entered one of the arms of the Y, it was removed from the system, the choice recorded, and the apparatus readied for another run.

Every possible precaution was taken to prevent at the point-of-choice any other imbalance of physical or chemical factors than that desired. The level of water in the two aquaria was kept identical at the beginning of every individual run so that during the run the pressure in the two arms of the Y at the point-of-choice was by necessity equal, the heads above them being equal. The temperature was kept the same in both aquaria (13.5–15° C.).

Before any experiments were made flow tests were conducted using colored indicators. Figure 2 shows the head of a worm at the point-of-choice (Fig. 1, B) with the flow characteristics superimposed upon it. If an indicator was put into one aquarium, it was clearly demonstrated that the flow characteristics were such that if a chemical were to be present in one aquarium, the head of the worm and its tentacles would be asymmetrically stimulated. The two streams did not mix completely at the point-of-choice, there being a region of high concentration of indicator at one

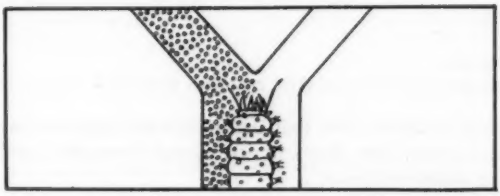

FIGURE 2. Enlargement of Figure 1, B. Flow characteristics as indicated by borax carmine, showing asymmetrical stimulation of tentacles of commensal at point-of-choice.

side, a narrow cone-shaped zone of mixing in the middle and a clear region without indicator at the opposite side. Farther down the stem and at the point of introduction (A) mixing was complete.

In addition, although worms were not strongly or negatively phototactic, in order to control differences in light as well as small differences in temperature, pressure, rate of flow or aeration, in any series of runs water from each aquarium was allowed to flow into alternate arms of the Y. For instance, if a series of runs were being made with a host in one aquarium and sea-water alone in the other, in the first run water from the host flowed into the left arm of the Y, in the next into the right arm, in the next into the left arm again, and so on alternately throughout the series. Between each and every individual run both arms were flushed out from the sea-water (or non-host) aquarium and between any series of tests the whole apparatus was thoroughly washed out with fresh sea-water. Both the glass and wooden aquaria were scrubbed and leached in fresh running sea-water for at least twelve hours. The wooden aquaria used to house hosts during runs were also periodically alternated.

OBSERVATIONS

In the data below the total number of times worms were placed in the test apparatus is recorded as the *"number of trials."*

If a worm moved up the stem of the Y and into either arm this is recorded as a *"run."*

If a worm failed within ten minutes from the time it was placed in the apparatus to reach the point-of-choice or enter either arm of the Y or if it reversed its position, this is recorded as a *"failure."*

The *"negative trials"* appearing in some of the tables below equal the sum of *"failures"* plus *"runs into the sea-water (or non-host) arm."*

Experiment No. 1. Are *Arctonoë fragilis* attracted by sea-water coming from an aquarium containing their host *Evasterias troschelii?*

No. of trials	43
No. of runs	35
No. of runs into *Evasterias* arm	33
No. of runs into sea-water arm	2
% runs into *Evasterias* arm	94.5%
Chi-square of choice (vs. 50/50)	27.4
P	$\ll .01$
No. of failures	8
% failures	18%
No. of worms used	13
No. of worms making five consecutive runs into *Evasterias* arm	5

It would appear from these data that the worms are attracted by some substance from the host aquarium and that they can distinguish between it and sea-water alone when they reach the point-of-choice.

Experiment No. 2. Is the ability of *A. fragilis* to determine which of the two arms of the Y contains sea-water from an *Evasterias* diminished if the other arm contains water from an aquarium containing a starfish of another genus, *Pisaster,* on which the worm is not commensal? Weight *Evasterias*—540 grams. Weight *Pisaster*—430 grams.

No. of trials	36
No. of runs	35
No. of runs into *Evasterias* arm	33
No. of runs into *Pisaster* arm	2
% runs into *Evasterias* arm	94.5%
Chi-square of choice	27.4
P	$\ll .01$
No. of failures	1
% failures	2%
No. of worms used	6
No. of worms making five consecutive runs into *Evasterias* arm	5

It would appear that the presence of the *Pisaster* in this experiment had no effect on the choice of the worms.

Experiment No. 3. What happens if in the above experiment we double the weight of non-host star in the other aquarium? Weight of host—489.5 grams. Weight of two *Pisaster*—866.1 grams.

No. of trials	37
No. of runs	36
No. of runs into *Evasterias* arm	31
No. of runs into *Pisaster* arm	5
% runs into *Evasterias* arm	86.1%
Chi-square of choice	18.68
P	$\ll .01$
No. of failures	1
% failures	2.7%
No. of worms used	6
No. of worms making five consecutive runs into *Evasterias* arm	4

It appears that doubling the weight of the non-host *Pisaster* had no significant effect on the ability of the worms to choose the arm of the Y in which water flowed from the host.

Combining all the above data for trials when the host was in the system:

No. of trials	116
No. of runs	106
No. of runs into *Evasterias* arm	97
No. of runs into sea-water or non-host arm	9
% runs into *Evasterias* arm	91.4%
Chi-square of choice	73.0
P	$\ll .01$
No. of failures	10
% failures	8.6%
No. of negative trials (9 plus 10)	19
No. of worms used	19
No. of worms making five consecutive runs into *Evasterias* arm	13

Experiment No. 4. How do *Arctonoë fragilis* collected from *Evasterias* behave when their own host is replaced in the test apparatus by a specimen of *Luidia foliolata* which is listed as an occasional host of *A. fragilis*? *Luidia*—weight approximately 920 grams.

No. of trials	35
No. of runs	14
No. of runs into *Luidia* arm	8
No. of runs into sea-water arm	6
% runs into *Luidia* arm	57%
Chi-square of choice	.285
P	.50–.95
No. of failures	21
% failures	72%
No. of negative trials (6 plus 21)	27
No. of worms used	9

It can be seen from the above that the distribution of runs into the *Luidia* arm and into the sea-water arm does not differ significantly from a chance distribution. Furthermore, if we compare the proportion of the runs into the *Luidia* arm to the negative trials (8/27) with the proportion, taken from the preceding sum of the data when the host was in the system, of the runs into the host arm to the negative trials (97/19), we see that the chi-square is 47.0 and the $P \ll .01$. It appears, therefore, that under these experimental conditions at least, *Luidia foliolata* has no attraction for the *A. fragilis* which are commensals of *Evasterias*, in spite of the fact that this worm has been described as occurring on *Luidia*.

Experiment No. 5. Are *Arctonoë* (*pulchra-fragilis*?) attracted by water coming from an aquarium containing their host *Luidia foliolata*? *Luidia*—weight approx. 920 grams.

No. of trials	35
No. of runs	20
No. of runs into *Luidia* arm	8
No. of runs into sea-water arm	12
% runs into *Luidia* arm	40%
Chi-square of choice	.80
P	.30–.50
No. of failures	15
No. of negative trials (15 plus 12)	27
No. of worms used	8

It appears that the worms cannot discern the presence of their host since the data for choice does not significantly differ from a chance distribution. That the behavior of the worms under these conditions is significantly different from that of worms in which there is evidence of a strong attraction can be seen if we compare the proportions of the number of runs into the host arm to the number of negative trials (8/27) with the same proportion for the sum of the data for *A. fragilis* and *Evasterias troschelii* (97/19). The chi-square is 47.0 and the $P \ll .01$.

Experiment No. 6. Can the above worms commensal with *Luidia* discern the presence in the system of the star *Evasterias* which has been shown to have a positive attraction for its own commensal *Arctonoë*?

No. of trials	18
No. of runs	5
No. of runs into *Evasterias* arm	2
No. of runs into sea-water arm	3
No. of failures	13
% failures	72%
No. of negative trials (13 plus 3)	16
No. of worms used	8

The very high proportion of negative trials to total trials (16/18) makes it clear that the substitution of an *Evasterias* for the host *Luidia* in the system has no effect on the behavior of these worms.

Experiment No. 7. Are *Arctonoë* (*pulchra-vittata*?) attracted by water coming from an aquarium containing their host *Stichopus californicus*? Host weight approx. 1000 gms.

No. of trials	56
No. of runs	50
No. of runs into *Stichopus* arm	45
No. of runs into sea-water arm	5
% runs into *Stichopus* arm	90%
Chi-square of choice	32.0
P	$\ll .01$
No. of failures	6
% failures	10.7%
No. of negative trials (6 plus 5)	11
No. of worms used	11

It would appear that these worms can clearly discern the presence of their host and distinguish between host-water and sea-water alone.

Experiment No. 8. Are *Arctonoë* commensal with *Stichopus* attracted by water from an aquarium containing another Holothurian, *Cucumaria miniata* (Brandt), found in the same general environment as their host? Combined weight of twelve *Cucumaria* in test aquarium—428 gms.

No. of trials	35
No. of runs	24
No. of runs into *Cucumaria* arm	9
No. of runs into sea-water arm	15
% runs into *Cucumaria* arm	37.5%
Chi-square of choice	1.5
P	.20–.30
No. of failures	11
% failures	31.5%
No. of negative trials (11 plus 15)	26
No. of worms used	6

It is clear that in this series of runs the distribution beyond the point-of-choice did not significantly differ from a chance one. That the behavior of the worms differed significantly from their behavior when the host was in the system can be seen when we compare the proportion of runs into the *Cucumaria* arm to negative trials (9/26) with the similar proportion from the preceding table when the host was in the system (45/11). The chi-square is 26.2 and the $P \ll .01$.

Experiment No. 9. What happens if, in the test apparatus, we cross hosts of two partnerships in which a clear attraction has been demonstrated? How do worms commensal with *Stichopus* behave if a specimen of *Evasterias troschelii* replaces their host in the system?

Series A. *Evasterias* weight approx. 425 grams

No. of trials	15
No. of runs	13
No. of runs into *Evasterias* arm	5
No. of runs into sea-water arm	8
Chi-square of choice	.68
P	.30–.50
No. of failures	2
No. of worms used	5

Series B. *Evasterias* weight approx. 950 grams

No. of trials	25
No. of runs	15
No. of runs into *Evasterias* arm	11
No. of runs into sea-water arm	4
Chi-square of choice	3.2
P	.05–.10
No. of failures	10
No. of worms used	5

It can be seen that in both series A and B the distribution beyond the point-of-choice did not significantly differ from a chance one. Comparing the proportions

of the number of runs into each arm in both sets of data we see that in series B in which the weight of *Evasterias* was increased in order to equal more nearly that of the host *Stichopus,* the runs into the *Evasterias* arm were in the majority. However, the number of failures (10) was high for an experiment demonstrating an attraction. A longer series of trials would have to be made in order to determine whether commensals from *Stichopus* may be significantly attracted by water from a large weight of their relative's host, *Evasterias.*

In order to compare the behavior of the worms in these two series of experiments with their behavior when the host was in the system, we may combine the data from series A and B.

No. of trials	40
No. of runs	28
No. of runs into *Evasterias* arm	16
No. of runs into sea-water arm	12
No. of failures	12
No. of negative trials (12 plus 12)	24
No. of worms used	10

That the behavior of the worms differed from their behavior when the host was in the system can be seen when we compare the proportion of runs into the *Evasterias* arm to negative trials (16/24) with the similar proportion when the host was in the system (45/11). Here the chi-square is 15.7 and the $P \ll .01$.

Experiment No. 10. How do worms commensal with *Evasterias* behave if a specimen of *Stichopus californicus* replaces their host in the system?:

No. of trials	35
No. of runs	33
No. of runs into *Stichopus* arm	17
No. of runs into sea-water arm	16
No. of failures	2
% failures	5.6%
No. of negative trials (2 plus 16)	18
No. of worms used	7

It is clear that in this experiment the data of choice did not differ from a chance distribution. That the behavior of the worms differed from their behavior when their host was in the system can be seen when we compare the proportion of runs into the *Stichopus* arm to negative trials (17/18) with the similar proportion from the sum of the data for experiments no. 1, 2 and 3 (97/19). Here the chi-square is 17.6 and the $P \ll .01$.

Discussion

The data above present clear statistical evidence that certain echinoderm hosts produce a specific diffusable substance that acts as a powerful attractant for their polychaete commensals. However, the data cannot describe the characteristic behavior of worms when they are under the influence of the attractant. Tested against sea-water alone, *A. fragilis* showed a random distribution of choice, but 8 of 21 trials were failures, and in the majority of the remainder of the runs the worms were sluggish, took the full ten minutes to reach the point-of-choice, and showed a

decided lack of interest in moving up the stem. This behavior was more or less characteristic of all tests when hosts were not in the system and of tests with the mud-star, *Luidia foliolata*. Tested against hosts, however, the characteristic behavior of worms was to hesitate for a brief period at the point of entrance and then suddenly to become activated and move up the stem, waving the head from side to side in searching movements. The delay at the point of entrance appeared in these experiments to be about the same time it took indicator to reach this point in the preliminary flow-tests.

In a number of experiments in which positive results were obtained it is clear that some of the runs into the sea-water or non-host arm were the result of the worm moving so swiftly up the stem of the Y under the influence of "host-factor" that without stopping at the point-of-choice to sample the streams, it simply moved into that arm of the Y nearest the side of the stem to which it was appressed when it reached the point-of-choice. A number of times during the course of the experiments worms were observed to move part way into the sea-water or non-host arm, then pull back and enter the host arm.

The failure of *Arctonoë* commensal with *Luidia* to respond to their own hosts is unexplained. However, it was impossible to obtain an entirely uninjured host for use in the test apparatus and if a whole one was put into the apparatus it soon autotomized one or more arms. Perhaps the failure to obtain a response by commensals depended upon the release of injury substances from these stars.

It would appear that other echinoderms not distantly related to hosts, but without commensals, have no attraction for the commensals. However, a survey of many more forms should be made before this can be certain.

Reciprocal experiments indicate that "host-factor" produced by the host of one partnership has no attraction for commensals of another partnership, in spite of the fact that the commensals may be extremely closely related. However, little light has been cast by these preliminary experiments on the interesting phenomenon of the restriction of what appear to be single commensal species to certain species only of a number of totally unrelated classes of hosts (see Table I). By similar tests it should be possible to give evidence whether or not attractants of two unrelated hosts of the same commensal species may be similar.

It is clearly apparent that details of the life-histories of the forms of the genus *Arctonoë* are needed, involving examination by specialists of extensive series of different age groups of commensals from each of the many host species. Although in the differentiation of the three forms it is almost certain that genetic factors are involved, it would be of great interest to determine whether or not the early stages of one form could be induced to develop on the host of another form and if so, whether morphological as well as physiological adaptations to the new host might occur independent of genetic factors. In certain species of insect parasites, structural polymorphism dependent upon host has been clearly demonstrated by Salt (1937) and others.

That organisms by undergoing their development in contact with certain specific chemical factors may be physiologically conditioned to respond positively to them has been demonstrated by Thorpe (1939) in his work with Drosophila. The work cast some light on earlier observations by Thorpe and Jones (1937) that the wasp *Nemeritis* (Hymenoptera: Ichneumonidae), normally a parasite of the Wax-moth *Ephestia*, could be conditioned to give a positive olfactory response to the Wax-

moth *Meliphora* if it had undergone its development on this abnormal host. Similar experiments could perhaps be conducted using marine partnerships; they might be initiated by using such forms as the polynoid *Halosydna brevisetosa* Kinberg, in which both free-living individuals and morphologically very different individuals commensal with certain terebellids are readily available. It is quite possible that just as in certain terrestrial host-parasite relationships (Thorpe and Jones, 1937) we have in these intimate marine associations the factor of olfactory conditioning acting as a mechanism of isolation, which Cushing (1941) suggests may be "a physiological factor acting upon population mechanics that does not depend upon genetic changes for changes in its specificity of action."

The general occurrence of chemical factors as controlling agents of host-commensal relationships in the sea must be surveyed by adapting our apparatus so that members of other phyla can be used, and finally an investigation must be made of the chemical nature of attractants.

Summary

1. Apparatus was developed which made it possible to determine whether commensals of the polynoid genus *Arctonoë* are attracted by specific substances to their hosts and whether they can distinguish between water coming from an aquarium containing their host and sea-water alone or water from non-host.

2. *Arctonoë fragilis* (Baird) commensal with the star *Evasterias troschelii* Stimpson are clearly attracted to their host and can distinguish between water coming from their host and sea-water alone. *Arctonoë* (*pulchra-vittata*?) commensal with the cucumber *Stichopus* are likewise attracted to their host.

3. *Arctonoë fragilis* commensal with *Evasterias* are not attracted to the non-host star, *Pisaster,* occurring in the same immediate environment as their host. *Arctonoë* (*pulchra-vittata*?) commensal with the cucumber *Stichopus* are likewise not attracted to the non-host *Cucumaria.*

4. *Arctonoë* (*pulchra-fragilis*?) commensal with the mud-star *Luidia* are not attracted, under these experimental conditions at least, to their own host. *Arctonoë fragilis* commensal with *Evasterias* are not attracted to the mud-star *Luidia,* in spite of the fact that *Luidia* may be a host of *fragilis.* These results are perhaps inconclusive, because injury substances may have been released into the apparatus by the mud-stars.

5. Cross experiments (*A. fragilis* from *Evasterias* tested against *Stichopus* and *A. pulchra-vittata* from *Stichopus* tested against *Evasterias*) indicate that attractants released by the two hosts are not the same, since neither commensal is attracted to its relative's host.

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THE EFFECTS OF SULFANILAMIDE UPON THE OXYGEN UPTAKE OF MITOTICALLY ACTIVE AND BLOCKED GRASSHOPPER EMBRYOS [1]

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The effects of sulfonamides upon bacteria have been repeatedly pointed out and various ideas as to their fundamental action in such cases have been advanced (Fildes, 1940; Woods and Fildes, 1940; Woods, 1940; Eyster, 1943; Steers and Sevag, 1949). That sulfonamides markedly interfere with such general cell activities as division and growth seems also well established (Fisher, Henry and Low, 1944). More specific effects of these reagents upon cellular enzyme systems have also been investigated and rather striking concepts of their modes of action demonstrated (Johnson and Chase, 1942; Mann and Keilin, 1940; Johnson, Eyring and Williams, 1942). Marked inhibitions of cell division, oxygen consumption and enzyme activity have been reported and of especial interest are the relations found between the effects of sulfanilamide upon oxygen uptake and cell division in various types of animal and plant cells (Fisher, Henry and Low, 1944). Fisher, Henry and Low (1944), for the sea urchin egg, have shown that when the cell division is stopped by sulfanilamide, the oxygen uptake and especially the activity system is also completely inhibited. In this respect the action of sulfanilamide seems to resemble that of certain narcotics (Johnson, 1942).

Since the embryo of the grasshopper in its development at constant temperature (25° C.) normally goes through a block or diapause period in which mitotic activity is completely stopped it becomes of some interest to determine the action of sulfanilamide on it during both the blocked and active phases of its development. Morphologically similar embryos in mitotically active and blocked states are readily available and lend themselves to such an investigation (Bodine and Boell, 1936). The present paper, therefore, deals with data concerning the effect of sulfanilamide upon the oxygen uptake of morphologically identical grasshopper embryos both in mitotically active and blocked conditions.

Material and Methods

Embryos, free from yolk, were obtained from eggs of the grasshopper, *Melanoplus differentialis*, as previously noted (Bodine and Boell, 1936). Oxygen determinations were carried out at 25° C. with Warburg manometers, using respiration flasks of 5 ml. capacity. Embryos were suspended in phosphate buffered (pH 6.8) NaCl solution (0.65 per cent) and solutions of sulfanilamide were also made up

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in it. Further details of procedure have already been described (Bodine and Fitzgerald, 1949).

Results

The effects of sulfanilamide, ranging in concentration from 0.005 M. to 0.06 M., on the oxygen uptake of both active and blocked embryos were determined. Results of many such experiments are graphically shown in Figure 1. An inspection of this figure shows that sulfanilamide depresses the rate of oxygen uptake of both active and blocked cells to approximately 50 per cent of the normal value. No

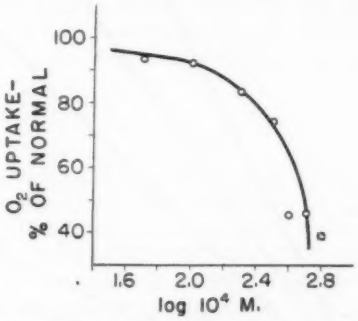

Figure 1. Shows the effect of sulfanilamide on the oxygen uptake of active and blocked embryos. Abscissa, log 10^4 M. sulfanilamide; ordinate, relative oxygen uptake rate, taking O_2 uptake of non-treated embryos as 100%. Circles represent averages of all experiments.

significant differences in responses between the mitotically active and blocked cells have been found (Table I). The effects of sulfanilamide upon the oxygen uptake do not seem to be conditioned by the mitotic activity of the cells of the embryos. By choosing embryos in various degrees of diapause or cellular block (as shown by rate of oxygen uptake) it is possible to show that the relative effects of sulfanilamide are similar in all cases. In Figure 2 are graphically shown the results of several experiments using 0.06 M. sulfanilamide and embryos in different levels of block. The degree of block or diapause in these embryos is indicated by their rates of oxygen uptake, i.e., embryos in complete block have much lower rates of oxygen consumption than similar ones out of block and actively developing (Bodine and

Table I

Inhibition of O_2 uptake in % of normal for embryos

Diapause	Postdiapause
44	70
48	55
69	58
68	74
65	43
49	
48	Av. = 60.0
56	
Av. = 57.2	

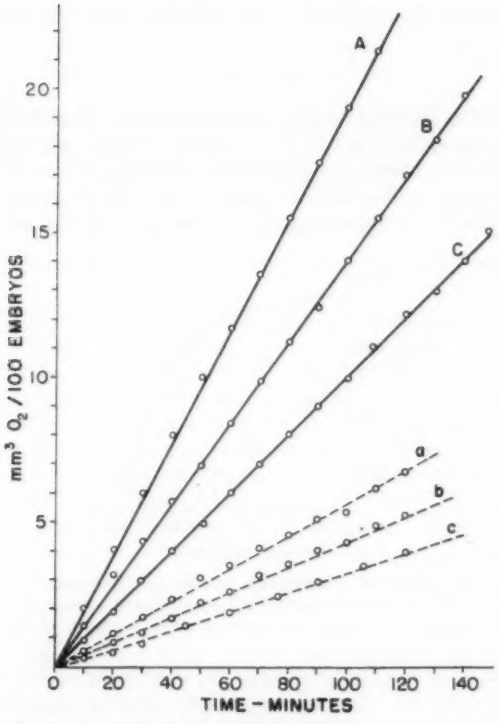

FIGURE 2. Shows effect of 0.06 M. sulfanilamide on the oxygen uptake of morphologically similar diapause and postdiapause embryos. Abscissa, time in minutes; Ordinate, mm.³ O_2/100 embryos. A = postdiapause embryos (2 days at 25° C.) in Ringer. a = same embryos exposed to sulfanilamide. B = embryos coming out of diapause. b = same exposed to drug. C = embryos in complete block or diapause. c = same exposed to drug.

Boell, 1936). An inspection of Figure 2 shows that for two-hour exposures to sulfanilamide the relative effects of the drug on the oxygen uptake of these embryos are practically constant throughout. It seems, therefore, that for the grasshopper embryo the particular states of their cellular activity have little to do with their response to sulfanilamide especially as regards their oxygen uptake. It is of some interest to note that the relative amounts of sulfanilamide sensitive (approx. 60–70 per cent) and sulfanilamide insensitive (approx. 30–40 per cent) fractions of the respiration seem to be practically identical in the mitotically active and blocked cells. The inhibitory effects of sulfanilamide for both types of embryos, except for prolonged exposures, are quite reversible.

DISCUSSION

The above results for the grasshopper embryo appear quite different from those found for the fertilized and unfertilized sea urchin egg (Fisher, Henry and Low, 1944). Fertilized sea urchin eggs behave quite like the grasshopper embryo while

unfertilized eggs seem to be quite resistant to the action of sulfanilamide. The relative degrees of inhibition of the oxygen uptake for the fertilized sea urchin egg and the grasshopper embryo are quite similar (55–60 per cent). No cytological examinations of sulfanilamide treated active grasshopper embryos have been made to determine possible cellular effects and especially as regards effects on cell division. However, the blocked grasshopper embryo contains no mitotically active cells when in true diapause (Slifer, 1932) and its respiratory rate perhaps represents a real basal one. To what extent one is justified in comparing the physiological differences in response to sulfanilamide of the sea urchin egg and the embryonic cells of the grasshopper is quite an open question. For the grasshopper embryo one may reasonably conclude, however, that the inhibitory effects of sulfanilamide on the oxygen uptake seem to be quite independent of the mitotic behavior of the embryonic cells.

Summary

1. The effects of sulfanilamide upon the oxygen uptake of mitotically active and blocked grasshopper embryos have been determined.

2. Sulfanilamide depresses the rate of oxygen consumption in both active and blocked embryos in a relatively similar fashion.

3. The sulfanilamide sensitive and insensitive fractions of the respiration are approximately constant for both active and blocked embryonic cells.

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DORSALIZATION OF THE VENTRAL MARGINAL ZONE OF THE TRITURUS GASTRULA. I. AMMONIA-TREATMENT OF THE MEDIO-VENTRAL MARGINAL ZONE[1]

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Introduction

In the early gastrula of amphibians the material for the notochord and mesoderm is localized between the animal and vegetative hemispheres in the form of a ring. This marginal zone is most extensive at its medio-dorsal section, which extends well above the equator, and represents the broadest section of the ring. As one proceeds from this part towards the opposite medio-ventral section, the ring becomes gradually narrower, its upper boundary gradually lower, and its narrowest and lowest limit, the medio-ventral section, lies opposite the future dorsal lip of the blastopore.

The distribution of the presumptive organ rudiments of the trunk mesoderm within this zone reflects the dorso-ventral sequence of the rudiments in the completed embryo. Medio-dorsally lies the presumptive notochord, on both sides of it the presumptive myotomes, which are followed by the presumptive pronephros and lateral plate, and finally the presumptive blood island, which occupies the medio-ventral section of the marginal zone.

This sequence of the prospective values within the marginal zone is again reflected in the mode of differentiation of the isolated sections of the marginal zone cultured in Holtfreter solution (Holtfreter, 1938). Although here the differentiation of the individual sections does not faithfully realize their prospective value, the dorso-ventral sequence of morphogenesis is in its general form clearly recognizable. The ventral section of the marginal zone differentiates blood island, nephric tubules and the derivatives of the lateral plate. However, neither notochord nor muscle is formed in the isolate from this section. In contrast to this the differentiation of the notochord and muscle is the predominant feature of the isolated dorsal section. It must be kept in mind, however, that especially in the dorsal section the "regulative" tendency is at work to develop more than the prospective value, so that a part of the explant from the dorsal section has always the chance to become one of the more ventral mesodermal rudiments or even ectodermal tissue.

If comparable sections of the trunk mesoderm are isolated later (at the beginning phase of neurulation) and cultured in epidermis vesicles, the individual sections can be distinguished more clearly through the mode of their differentiation (Yamada, 1939, 1940). But even in this case the prospective value is not always

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faithfully followed. Further, it is demonstrated that by adding a piece of the presumptive notochord to the individual sections of the mesoderm of the neurula it is possible to alter the mode of differentiation of each isolate. This effect of the notochord consists in shifting the mode of differentiation of the isolate toward the dorsal side. The presumptive blood island which differentiates blood island, mesothelium and blood vessels in isolation, gives rise to nephric tubules and only a small quantity of blood cells, if it is combined with notochord material. The presumptive pronephros, which develops, in isolation, nephric tubules and blood cells, shows clear differentiation of muscle cells in the direct vicinity of the implanted notochord tissue. Presumptive myotome, which in isolation gives rise to nephric tubules besides undifferentiated myoblasts, develops muscle cells with definite fibrils on implantation of the notochord material. Thus the effect of the notochord on the morphogenesis of the mesoderm can be designated as "dorsalization." From these results it may be expected that also the isolated ventral marginal zone of the gastrula could be dorsalized by appropriate methods, perhaps with more profound results, since the material must be more plastic in the gastrula than in the neurula.

It is well known that the presumptive ectoderm of the early gastrula reacts to many chemical stimuli by undergoing neural differentiation. The most typical case may be the neuralization of the isolated gastrula ectoderm caused by a sublethal cytolysis, as demonstrated by Holtfreter (1945, 1947). Through brief treatment of the isolated gastrula ectoderm with a solution of extreme pH (higher than 9.4 or lower than 5.0), Ca-free Holtfreter solution, distilled water and 10 per cent alcohol, he obtained differentiation of brain, eye, placodes, mesenchyme and melanophores. Thus the cephalic ectodermal structures and mesectodermal components were induced, while the more caudal ectodermal structures, such as the otocyst and spinal cord, and the mesodermal structures were wholly absent. The arrangement and form of the induced rudiments were, however, highly atypical. The same seems to hold also for "self-induced" explants obtained by Barth (1942) and Holtfreter (1944) and for the explants of Shen (1942) cultured in a dilute solution of a hydrocarbon. Morphogenetic response of the isolated ectoderm to implantation of a protein belongs also to this category (Yamada, 1947). Here the induced structures are more diversified and typically organized than in the above cases.

All these inductions of the neural differentiation in the ectoderm are dorsalizations as defined above. This may become clear if one recalls the fact that epidermal differentiation is a ventral mode of differentiation, while brain differentiation is a dorsal one, according to the topographical relation which can be visualized, for instance, in a cross section of the anterior part of an early neurula. Between two extreme levels lie the intermediate levels of the sense organs, placodes and mesectoderm. Together they constitute the dorso-ventral scale of the anterior ectoderm. Induction or neuralization is then nothing but the conversion of the ventral mode into a more dorsal one. Thus the sublethal cytolysis dorsalizes the isolated ectoderm, which untreated tends to adopt the ventral mode of differentiation. Now in the double-potential theory proposed by the present author (Yamada, 1947, see below) the dorso-ventrality of the morphogenesis has a common basis in the ectoderm as well as in the mesoderm, and consequently the internal factor which regulates it in normal development may be of the same quality throughout the embryo. From this it may be expected that any factor which has a dorsalizing effect on the competent ectoderm can dorsalize the development of the mesoderm,

if it is given in an appropriate condition. This theoretical expectation is tested in the following experiments.

As the dorsalizing procedure a brief exposure to a solution of ammonia was adopted. At first this treatment was tested on the isolated ectoderm of the gastrula of *Triturus pyrrhogaster* (control series I), and after its dorsalizing effect was ascertained, the isolated medio-ventral marginal zone of the gastrula of the same species was treated in the same way (experimental series). Parallel to this series, the untreated medio-ventral and latero-ventral marginal zones were isolated and cultured as the control of the reacting system (control series II).

Ammonia was used in the experiment since it actually is concentrated in the dorsal regions of the amphibian embryo during the phase of gastrulation and neurulation (Boell, Needham and Rogers, 1939), and may have the role of a mediating factor in morphogenetic induction.

Material and Technique

The eggs of *Triturus pyrrhogaster* were obtained through implantation of ox hypophysis in the mature female, and fertilized naturally or artificially. Operations and culture were done under sterile conditions. The egg capsule was washed with 60 per cent alcohol for 50 seconds, thoroughly rinsed in sterile Holtfreter solution and then removed with fine scissors sterilized with dry heat. After freeing from the vitelline membrane with forceps, the embryos were operated on with a glass needle on the Schotté ring (Yamada, 1937). Standard Holtfreter's solution (pH 7.2–7.6) was used exclusively for operation and culture. Sodium bicarbonate for the Holtfreter solution was sterilized with 60 per cent alcohol and after drying dissolved in sterile distilled water and then added to the unbuffered solution which had been boiled and cooled beforehand. The solution of ammonia used for the shock treatment was prepared immediately before use by adding 0.01 cc. of 20 per cent ammonia solution to 10 cc. of Holtfreter solution. Its pH was roughly estimated to be 11.8–12.2. The condition of the laboratory did not permit the regulation of culture temperatures which fluctuated between 12° and 26° C. However, no essential effect was noted other than a variation in the velocity of development. The isolates were cultured in covered glass dishes of 25 cc. volume. After culturing for seven to eighteen days the isolates were fixed in Bouin's fluid, stained in bulk with borax carmine, sectioned at 12 μ and stained with picro—"Blauschwarz."

The developmental stage of the operated embryo was classified according to the following criteria: Stage I: The youngest gastrula with the first sign of the blastopore as a pigmented patch; Stage II: Gastrula shortly after formation of groove of invagination; Stage III: The blastopore crescent-shaped.

Control Series I: Shock Treatment of the Isolated Gastrula Ectoderm with Ammonia

It was expected that the isolated ectoderm of the early gastrula of *Triturus pyrrhogaster* would differentiate cephalic ectodermal structures if exposed temporarily to a solution of ammonia, just like the isolated ectoderm of *Triturus torosus* (Holtfreter, 1945, 1947).

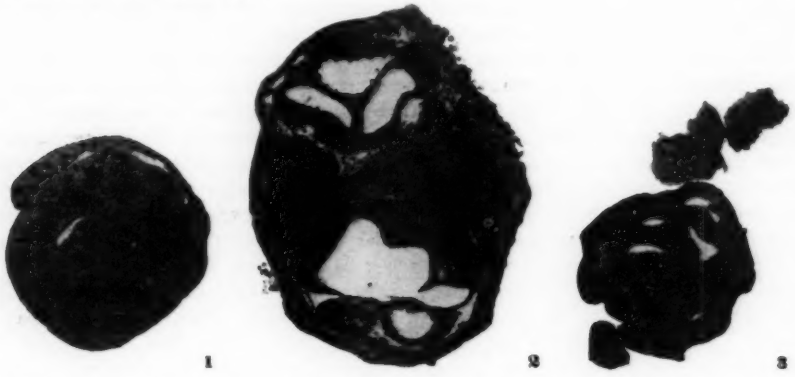
The presumptive ectoderm of the early gastrula was excised and put in the dilute solution of ammonia (see above). Special caution was taken not to include

in the isolate any cells from the marginal zone. After a latent period of ca. 50 seconds the explant began to disintegrate. The first sign was disruption and break-down of the pigmented surface into small fragments. This process was often accompanied by inverse curling of the explant edge, which resulted in the formation of a cup with the original internal surface occupying the convex outside. This cup persisted only for a while. Then the individual cells rounded up, became transparent and swollen and burst out of the aggregate. As the disaggregation progressed further a sticky transparent colloid was released. Thus within three to four minutes the original contour of the explants was almost lost. The velocity of the disintegration varied considerably, perhaps owing to the differences in the state of the curling and size of the explants. Before the explants were completely disaggregated they were transferred to another dish containing Holtfreter solution. Here the cells became less and less transparent and a part of them adhered to each other to form a solid aggregate of varying size, while other cells remained free on the bottom of the dish and were later discarded. Five explants were treated simultaneously with ammonia solution, and the number of the resulting aggregates varied from two to three. An aggregation of cells from two different isolates to form a common aggregate was a usual occurrence. The solid aggregate was then further transferred to the second dish with Holtfreter solution and cultured as usual. The surface of the aggregate was almost perfectly free of pigment and seemed to be occupied by the originally internal surface of the ectoderm. In the course of the subsequent three days a considerable number of cells were discharged from the reaggregated explant. In most cases the latter became a more or less regular, compact spheroid. No extensive adhesion to the glass bottom was observed. The formation of irregular small folds which are characteristic for the untreated ectoderm explant of this species occurred only in four of forty explants examined. On the other hand, no neural plate could be observed on the surface of our aggregates. This deserves attention in so far as the isolated ectoderm of this species shows a very clear neural plate under the inductive influence of chemicals and the organizer. However, by dissecting the aggregates two to three days after the ammonia-treatment, small strongly pigmented areas were found in its interior, scattered among the pigment-free cells. These areas are derived perhaps from the original surface of the ectoderm and have the significance of a neural plate. Probably neural differentiation originates in connection with them.

Of forty microscopically examined explants, thirty-one (77 per cent) showed neural differentiation. Four of the nine without neural differentiation belonged to the afore-mentioned type with many irregular folds. In sections, they cannot be distinguished from the untreated explant of *Triturus* ectoderm, being an unorganized mass of epidermis cells. The five remaining explants without neural differentiation are spherical in shape and consist of tightly condensed epidermal cells. Within them are encountered many cysts which often enclose cytolyzed cell masses besides densely pigmented cells, suggesting severe disturbance of the metabolism.

The structures encountered in the neuralized explants may be classified as brain-type, eye-type, neural mass, nose-type, lentoid, atypical neural balls, mesenchyme, chromatophores and frontal glands. It must be added, however, that the classification is of a rather provisional character and does not mean necessarily the definitive identity of the induced structure with the corresponding normal rudiments (see below).

Brain-type. Of twenty-nine brain-like bodies three show a rather normal form (Fig. 1). The typical appearance is often emphasized by the presence of nasal placodes closely applied to the "brain." In the other brain-like bodies the morphology is atypical in different degrees. The most atypical state is attained when an enormous body is furnished with a number of highly irregular lumina. One of six such cases is shown in Figure 2.



FIGURES 1–3. Neural tissues induced in the isolated ectoderm through exposure to ammonia solution. Fig. 1. A brain-like structure with a "nasal placode" on the right side. Fig. 2. A large explant showing two extensive brain-like structures with many lumina, accompanied by some placodes and mesenchyme. Fig. 3. An irregular brain-like vesicle accompanied by epidermal balls with frontal glands. × ca. 60.

Eye-type. In only two cases eye-like tissue is developed in connection with the brain-type differentiation. One of them is provided with a pigment layer, while the other is accompanied by a lentoid.

Neural mass. A group of neuralized cells without definite morphological characterization. This occurs regularly when small explants are perfectly neuralized and no epidermis exists to cover the neural tissue. Seven such cases are recorded.

Neural balls. This category also has no corresponding normal structure. It is probably heterogeneous, comprising some neural vesicles which are too small to be called a brain, and some bodies of placodal nature often enclosing large pigment-vacuoles. Eleven neural balls are differentiated in eight explants.

Nose-type. Of eighteen nasal placodes obtained, thirteen are closely applied to the brain and show a fairly normal condition (Fig. 1). Two others are differentiated without accompanying neural tissue.

Mesenchyme and melanophores. These elements are differentiated definitely only in one explant in close relation to the induced neural tissue. They cannot be distinguished from the normal ones.

Frontal glands. Typically differentiated glands are found in nine explants. They often form irregular groups with epidermal cells and are attached to the neuralized cell-mass as a protuberance (Fig. 3).

The size of the explants varied from 0.32×0.27 mm. to 1.28×1.05 mm. (expressed in the major and minor axes of the fixed explants, which are assumed

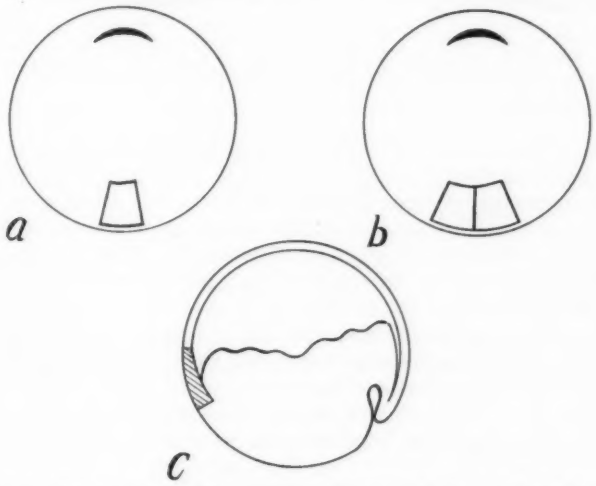
to be an ellipsoid). Neuralization occurred in the smallest as well as in the largest ones. Often the smaller explants are perfectly neuralized, while in the larger ones the morphogenesis of the induced structures progressed better. In six cases all the cells of the aggregate are neuralized so that the neural tissues are exposed on all sides to the medium. But even in such a state some lumina developed inside the neural mass and endowed it with a brain-like appearance (Fig. 3).

Summarizing, we can say that through the ammonia-treatment of the gastrula ectoderm only cephalic ectodermal or mesectodermal differentiations are induced, while the mesodermal or relatively caudal ectodermal differentiations are wholly absent. The results conform well with the findings of Holtfreter (1944a, 1947).

It may be added that the same treatment was carried out on a number of isolated pieces of ectoderm from the late gastrula and early neurula. They were all underlain with endomesoderm. Most of them did not recover from the disintegration. Only two explants from the presumptive epidermis of the gastrula with large yolk-plug were successfully cultured. Upon examination in sections, one of them shows the brain-type and the other the nose-type differentiation.

Control Series II: Differentiation of the Isolated Ventral Marginal Zone

A part of the ventral marginal zone from the early gastrula was isolated and cultured in Holtfreter solution. Two different ways were adopted to obtain the isolate. In series IIa two symmetrical sectors of the marginal zone were extended ca. 20° from the ventral median line on both sides (latero-ventral section), while in series IIb the sector of 20° lying exactly medio-ventrally was isolated (Fig. 4a–c). Utmost caution was taken against inclusion of the cells from the adjacent



Figures 4a–c. Scheme of operation. The early gastrula, in *a* and *b* viewed from the vegetative pole, in *c* shown in the median section. Fig. 4a. Operation for the "medio-ventral" section of the marginal zone. Fig. 4b. Operation for the "latero-ventral" section of the marginal zone.

sectors of the marginal zone in the isolate. The isolated pieces contained however, besides the cells of the marginal zone, some cells from the adjoining ectoderm and endoderm. This inclusion was intentional because for a good histological differentiation of mesoderm cells the presence of cells from other germ layers, which later furnish a covering tissue, is a necessary condition. The isolated piece was in most cases fused with other pieces into a single explant and cultured. The number of pieces constituting one explant varied from one to six. Several examples will be described:

VRK 108. From five gastrulae of stage II the medio-ventral sectors of the marginal zone were isolated and all were fused together in an explant and cultured in Holtfreter solution. One day after the operation the endo-mesodermal part of the explant formed a large whitish hemisphere, on which five ectodermal parts were attached separately as small cones. On the following days the ectodermal com-

FIGURES 5 AND 6. Development of the isolated ventral marginal zone. Fig. 5. Differentiation of blood island (in the center), enclosed in the mesothelium, externally covered by the epidermis. Fig. 6. Blood cells, blood vessels, and mesenchyme besides epidermal covering and undifferentiated endoderm. × ca. 60.

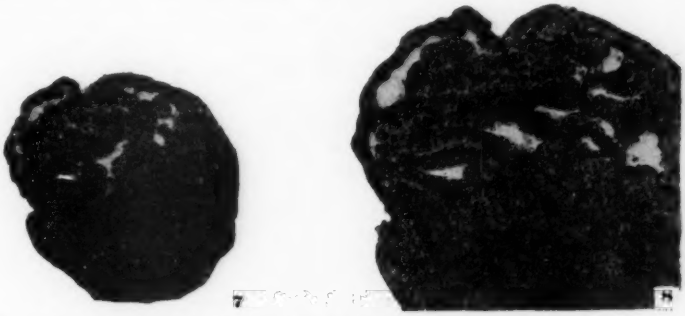
ponents gradually covered the surface of the endo-mesodermal hemisphere, and four of them fused with each other without losing their original individuality. Twelve days after the operation (culture temperature 21°–26° C.), when the large combined explant was fixed, it was found attached to the glass bottom with a cylinder of naked endoderm from which protruded a somewhat irregular, elongated body. It was covered completely with the epidermis, half-transparent and expanded. The size of the explant: 1.66 mm. × 1.28 mm.

Microscopical examination reveals that the protruded part of the explant is filled with loose mesodermal tissues: the yolk-rich mesenchyme, blood vessels enclosing blood cells and the mesothelium lining a wide space which is filled with blood cells (Fig. 6). Within the meshwork of mesenchyme and blood vessels is differentiated a piece of pronephric tubule, with one end attached to the endodermal cylinder.

VRK 5. From two gastrulae of stage II the medio-ventral sectors were cut out, fused together and cultured in Holtfreter solution. In the course of two days the explant formed a smaller compact sphere attached loosely to the bottom of the

culture dish. Cultured for fifteen days at 18°–23° C., the size of the explant: 0.84 × 0.78 mm.

Microscopical study shows that ca. three-quarters of the external surface of the explant is covered by endodermal epithelium. The latter is replaced by a well-differentiated mesodermal epithelium on the remaining surface. Within the mesodermal epithelium a cavity is formed which encloses a large amount of highly entangled pronephric tubules (Fig. 7). The tubules are connected with the external epithelium at some points. There exists no clear boundary between the tubules and epithelium and they both show a similar histological construction (Fig. 8).



FIGURES 7 AND 8. Differentiation of nephric tubules out of the isolated ventral marginal zone. A quantity of yolk-rich endoderm, no ectodermal component. Fig. 7, × ca. 60; Fig. 8, × ca. 100.

Obviously the external epithelium is nothing but the pronephric tissue modified by the topographical relation. Some sections of the tubules are free of yolk and better differentiated, while the others are laden with much yolk and seem to be retarded in differentiation. In all sections the central canal is poorly developed. A small quantity of mesenchyme, blood cells and melanophores are differentiated among the tubules. Embedded in the endoderm are some concentrated groups of mesoderm cells without any clear differentiation.

VRK a'. Operated as in the last example and cultured for eleven days at 19°–22° C. The explant developed into a spherical vesicle covered by the epidermis. Later two protuberances were formed at each pole, one elongated, the other spheroidal in shape. The size of the explant: 1.24 × 0.73 mm.

Microscopical study reveals that the main part of the highly expanded explant is filled with a large amount of blood cells enclosed in a cavity lined with mesothelium (Fig. 5). In the narrow space between the mesothelium and the epidermis are encountered a smaller quantity of blood cells, blood vessels, mesenchyme and a few melanophores. Each of the above-mentioned protuberances contains a part consisting of endodermal cells without any histogenesis.

The behavior of the explants as observed in the living condition may be summarized as follows. During the first several hours after isolation the explant acquired a more or less spherical form with expulsion of a small number of free cells. In the subsequent one to two days the pigmented ectodermal part of the explant became separated from the endo-mesodermal part by developing a promi-

nence which exhibited form changes of considerable degree. Thereafter the prominence retreated and the ectodermal part gradually fused to the endo-mesodermal ball, covering it more or less perfectly. In some cases the ectodermal prominence was constricted away from the endo-mesodermal part. Further morphogenesis of the explant is variable. It may develop a spheroidal compact body as in the case of the above described *VRK 5*, or a free spheroidal body covered by the epidermis as in the case of *VRK a'*, or in still other cases, a flat discoidal body firmly attached to the bottom and either covered or not covered by the epidermis. After the establishment of any of these forms further change in the external form was infrequent, except the enlargement through uptake of water from without, which often resulted in more or less transparent explants. In one case a minute tail-like formation developed from a discoidal explant (see below).

The results of the histological study of seventy-nine available cases are summarized in Table I. The explanted mesoderm has developed the following tissues: blood cells, pronephric tubules, mesothelium, blood vessels, mesenchyme and possi-

TABLE I

Differentiation of the isolated ventral marginal zone

Origin of explants	Number of explants	Frequency of tissues differentiated in the explants							
		Blood cells	Meso-thelium	Blood vessels	Mesen-chyme I	Mesen-chyme II	Melano-phores	Nephric tubules	"Somite"
Lateroventral (IIa)	44	34 77%	11 25%	9 20%	4 9%	3 6%	8 18%	25 56%	2 4%
Medioventral (IIb)	35	22 62%	6 17%	7 20%	7 20%	1 2%	1 2%	20 57%	1 2%
Total	79	56 70%	17 21%	16 20%	11 13%	4 5%	9 11%	45 56%	3 3%

bly melanophores. The table shows clearly that no significant difference can be recognized in the frequency of these tissues between series IIa and IIb (Table II). Therefore we can neglect for the moment this difference in operation and treat IIa and IIb together.

Blood cells. In most cases these occur in large numbers in a condensed group comparable to the normal blood island. In eighteen cases the blood cells are found in a voluminous cavity lined with mesothelium (Fig. 5). In twenty-four cases they are differentiated in a restricted space developed within the endoderm, or between the endoderm and the bottom of the culture dish. In fourteen other cases blood cells are gathered in a smaller group between mesenchyme or nephric tubules.

Nephric tubules. Tubules occur in the majority of cases in a restricted space between endoderm mass, or completely embedded in the latter, or (rarely) in the mesh-work of the mesenchyme and blood vessels. When they are perfectly embedded in the endoderm their histogenesis is very poor, but when they are bathed in the fluid of a cavity and invested with mesenchyme and blood vessels their histogenesis is more typical.

TABLE II

Significance of the origin of explants (medio-ventral or latero-ventral) and the number of pieces fused together for the differentiation of explants from the marginal zone

Origin of explants	Number of pieces fused together	Number of explants	Principal differentiations		
			Blood cells	Nephric tubules	"Somite"
Lateroventral (IIa)	2	44	34 77%	25 56%	2 4%
Medioventral (IIb)	1–2	26	15 58%	15 58%	1 3%
	5–6	9	7 77%	5 55%	0
Total		79	56 70%	45 56%	3 3%

It is interesting to note that between the blood cells and tubules the following relation exists:

(a) explants with blood cells but without tubules.....34 cases (43%)
(b) explants with tubules but without blood cells.....23 cases (29%)
(c) explants with both of them.....22 cases (27%)

Thus, all the explants have developed either one or both structures. The blood cells and tubules can be considered, therefore, as the main differentiations of the explants.

Mesothelium. A thin epithelium, lining rather voluminous cavities and developed within the epidermis, is considered under this heading. It is accompanied in most cases by blood cells, and often by blood vessels. In sections through explants with such a cavity, a colloidal constituent is precipitated in the form of finer granules or reticulum, which is stained intensively with "Blauschwarz." Quite probably the cavity formation is the result of the release of colloid from the mesothelium cells or mesenchyme cells.

Mesenchyme. Under mesenchyme two defined groups are encountered. One is characterized by large, yolk-laden cytoplasm and a very coarse mesh-work which is stained only slightly with "Blauschwarz" ("mesenchyme I" in Table I). The other is characterized by the yolk-free cytoplasm and slender processes intensively stained with "Blauschwarz" ("mesenchyme II" in Table I). In one explant in which the limb-bud is developed, mesenchyme II occurs in large quantities. Type I is far more frequent than type II and is observed mostly in association with blood vessels and blood cells.

Blood vessel. Blood vessels are formed either within the mesh-work of mesenchyme (Fig. 6) or just beside the mesothelium. Apparently they do not form a perfectly closed system. Blood cells are not always found within the vessels.

Melanophores. Out of nine explants with melanophores three do not possess an ectodermal component. In these cases, at least, it is probable that the melanophores are derived from the marginal zone cells and not induced from the ectoderm as a mesectodermal component.

Myoblasts. In a few cases a small part of the explanted mesoderm gave rise to a group of elongated cells which have the features of a myoblast. But in not a single case are definitive muscle cells with myofibrils differentiated. More often, condensed mesodermal cells are found in connection with the pronephric tubules. They may be considered as a fragment of an early somite before the myotome is segregated from another part of it. However, the differentiation did not progress further and we cannot designate these cells even as myoblasts.

Tail-like process. The above-mentioned minute process which occurred only in one explant, reveals itself in section as a very small tail-like process filled with mesenchyme II. No axial structures are found in it. But proximal to the process three small groups of mesoderm cells are found. One of them is composed of mesenchyme cells which are in the process of breaking up into free cells (of type II). Two remaining groups are compact but have apparently the same fate as the first. In none of the three groups is a differentiation of myoblasts discernible. Taken as a whole, the structure may be considered an incomplete tail without axial tissue. In other parts of this explant the mesoderm has differentiated nephric tubules and a small amount of blood cells.

Summarizing the observations of this series, it may be said that both the isolated medio-ventral and latero-ventral sectors of the marginal zone differentiate in the main: blood islands, nephric tubules and derivatives of the lateral plate. But in a very restricted number of cases (3.7 per cent) and in restricted quantity, some features of somitic development are observed, in no case, however, leading to complete differentiation of a myotome. Not a single explant differentiated notochord or definite muscle cells.

Table II shows further that the difference in the number of the pieces fused together did not have an important effect on the outcome of the differentiation so far as the present investigation is concerned. But from these restricted results it is not permissible to draw any general conclusions as to the relation between the quantity of the material and the quality of differentiation.

Comparing the differentiation of the explant with its prospective value, the following may be pointed out. The differentiation of blood island, mesothelium, blood vessels and probably also of mesenchyme I corresponds to the prospective value of the isolated region. But it is rather questionable whether the nephric tubules are contained in the prospective value of this section of the marginal zone, as maintained earlier by Holtfreter (1938). The somitic development which occurs very seldom and incompletely is surely not in accord with the prospective value of the isolate. Thus we must conclude that the main feature of development of the explanted ventral marginal zone corresponds to its prospective value, although there is a recognizable tendency to differentiate structures which lie more dorsally in the dorso-ventral scale of trunk mesoderm differentiation than the prospective value of the material.

Experimental Series: Shock Treatment of the Isolated Ventral Marginal Zone with Ammonia

In this series only the medio-ventral sector of the marginal zone as defined above (Fig. 4) was used as the material. The isolate, prepared in the same way as in the last series, was treated with 0.02 per cent ammonia solution until the disinte-

gration of the isolate took place, then transferred to Holtfreter solution where a part of the cells reaggregated. In this case more time was required for the disintegration than in control series I. This is perhaps due to the presence of a thick layer of yolk cells within the isolate, which are apparently more resistant to the dispersive action of the medium and protect the superficial layer of mesoderm and ectoderm from the action. An attempt was made to stop the treatment at a state of disintegration, which was comparable to that attained in the control series I. This was realized, however, only approximately, as the variability in the velocity and detailed course of the disintegration process was unexpectedly high. The period of treatment, which was determined according to the morphological state of disintegration, varied between 3½ to 7 minutes. One aggregate was obtained from a single original isolate, or from a number of original isolates. In the latter case the original isolates were treated with ammonia and the disintegrating masses were fused into one mass with the aid of a glass needle and transferred to a dish containing Holtfreter solution. Without further manipulation the cells reaggregated into a single body. In this way, two to ten original isolates were fused into a single aggregate. However, it must be kept in mind that the quantity of the resulting explant is much smaller than the sum of the original isolates, as a severe loss of cells occurs during and after treatment.

The morphological aspect of the course of disintegration of the marginal zone in ammonia solution, differed from that of the ectoderm in that the curling was insignificant and the process of actual disintegration was foreshadowed by a slow but steady rise in transparency of the cells. Some examples follow:

AVR 92. From four gastrulae of stage I, the medio-ventral piece of the marginal zone was isolated and treated with 0.02 per cent ammonia solution for ca. 4 minutes until the original contour of the individual isolates was almost lost, then transferred to Holtfreter solution. A severe loss of cells was observed at the time of treatment and during subsequent days, resulting in reduction of the size of aggregates. Large white endoderm cells, light brown mesoderm cells and dark pigmented ectoderm cells were discharged. Five days after the treatment the ovoid aggregate showed a small dark groove on one of its poles, while the rest of the surface was covered by the opaque, white endoderm. The explant adhered loosely to the bottom of the culture dish. Cultured for fifteen days at a temperature of 14°–24° C., the size of the explant: 1.81 × 0.90 mm.

Microscopic observations: Covered by an undifferentiated endoderm layer, a large complex of notochord and muscle developed (Figs. 9, 10). In one-half of the explant the notochord is only slightly curved and approximates the normal notochord of the corresponding stage in the diameter (0.15 mm.), while in the other half the notochord takes an irregular complex course and shows varying cross sections. Most of the notochord is composed of perfectly vacuolated cells and provided with a layer of connective tissue cells. The muscle tissue which accompanies the notochord is regularly segmented where the course of the notochord is more or less straight, but irregularly arranged where the course of the latter is complicated. The muscle tissue is provided with clearly differentiated myofibrils and a small amount of yolk granules, and invested with a layer of connective tissue cells, which seem to be derived from the original somite. Some elongated neural bodies are formed among the rather irregular complex of notochord and muscle. In some of its parts the neural tissue is laden with exceptionally abundant yolk granules,

and does not have a definite boundary against the neighboring mesoderm. It is difficult to identify the neural structure as any part of the normal nervous system, but as it is stretched in a slender form one can perhaps ascribe to it a caudal nature. A small group of the pronephric tubules are formed between muscle, notochord and endoderm. A mesodermal protuberance exposed to the medium is formed. This looks like a limb-bud, but is partly continuous with a fragment of neural tissue.

9 10

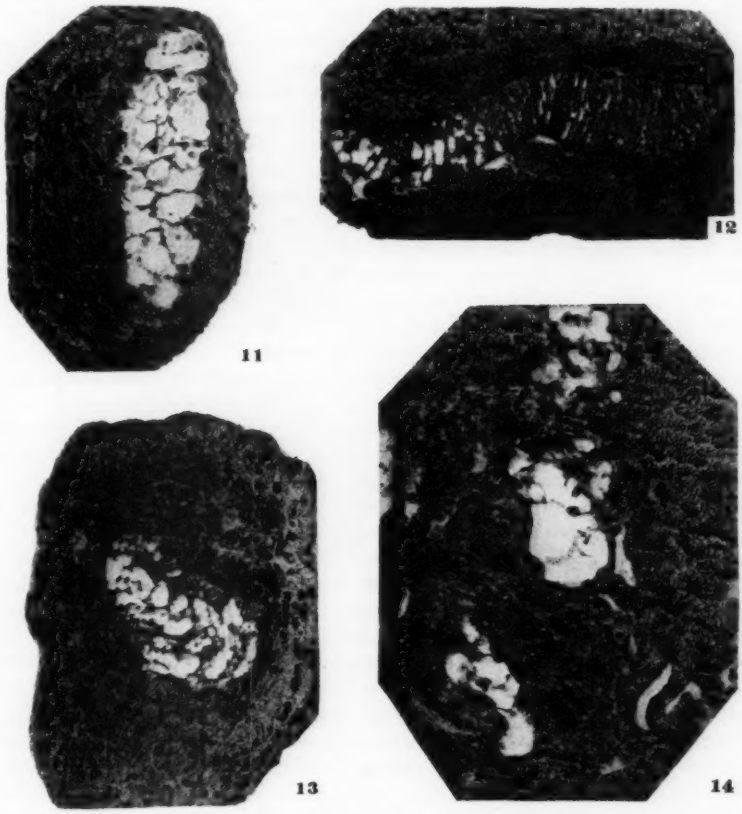
FIGURES 9 AND 10. Differentiation of notochord, muscle and neural tissue from the isolated ventral marginal zone after exposure to ammonia solution. Two sections through the same explant. Fig. 9. Notochord (right) and muscle (left) enclosed in the endoderm. Fig. 10. Somewhat irregular notochord accompanied by the muscle (left) and neural tissue (below). × ca. 60.

AVR 152. Three pieces of the medio-ventral section of the marginal zone were isolated from gastrulae of stages I and II and treated with ammonia solution for ca. 3½ minutes. Before the disintegration set in they were put back into Holtfreter solution. The aggregate formed a somewhat deformed sphere with small pigmented cell-groups protruding from its otherwise smooth whitish surface. An appreciable quantity of cells were discharged. Cultured eight days at 22°–24° C., the size of explant: 1.40 × 0.65 mm.

Sections through the explant show that the center of the ovoid explant is occupied by a piece of notochord with very irregular contour (Fig. 13). The maximal diameter is found to be 0.17 mm., a value which exceeds the value of a normal notochord of the corresponding stage. Some of the notochord cells are in an earlier phase of the vacuolization showing numerous small vacuoles within the cytoplasm, while others are completely vacuolated, retaining a minimal amount of cytoplasm. The notochord is incompletely surrounded by myotomes. In the periphery of each myoblast, the myofibrils are differentiated and enclose internally a good quantity of yolk granules. In some parts of the explant both tissues, the notochord and myotome, are not perfectly delimited, having a transitional zone in which the cells show an intermediate character. To one end of the notochord is attached a piece of neural tissue which is devoid of a definite structure, and is laden with abundant yolk. Some of the myotomes are connected with this neural tissue

without clear boundary. The external covering of the explant is chiefly a layer of endoderm.

At the pointed end of the explant the covering is ruptured by the above mentioned neural tissue with one extremity of the notochord embedded in it. The myotomes are directly applied to the endodermal covering, except in the region where a group of nephric tubules are formed between them. No blood island or mesothelium is differentiated.



FIGURES 11–14. Different types of notochord induced in the ventral marginal zone through ammonia-treatment. Fig. 11. A straight cord composed of perfectly vacuolized cells, accompanied by myotomes on the left side. Fig. 12. A typical young notochord, in which the vacuolization has progressed farther toward one end (left in the picture) than toward the other. Fig. 13. Rather atypical differentiation of the notochord, not clearly delimited from the surrounding myotomes (above and below) and neural cells (right). Fig. 14. In the upper third of the figure notochord cells are scattered within a field of neuralized cells and myoblasts. In the lower two-thirds an entangled notochord is formed within the muscle tissue. At right, below nephric tubules. All figures × ca. 100.

AVR 32. Three pieces of the medio-ventral marginal zone were isolated from gastrulae of stage I and immersed in ammonia solution. The treatment was continued until the isolates began to disintegrate into separate cells (ca. 5 minutes). In Holtfreter solution, the cells aggregated into an elongated, compact group which, in the course of cultivation, adhered loosely to the bottom. A rather small number of cells were set free. When the aggregate was fixed in Bouin's fluid after thirteen days of culture at a very low temperature (12°–22° C.), it was covered by a whitish layer of endoderm, through which an elongated dark field was vaguely visible. The size of explant: 1.78 × 0.89 mm.

Microscopic observations: A large piece of notochord (length ca. 1.2 mm.) runs along the long axis of the explant. Consistent with the low temperature it shows the first sign of its histogenesis. One end of this piece, which may be called "caudal" because of its delayed histogenesis, is twisted in a spiral, with its highly flattened, yolk-laden cells arranged in a single row like a pile of coins. The cytoplasm is almost devoid of vacuoles. The middle part of the piece is almost straight (Fig. 12). The arrangement of flattened cells in a single row is very accentuated and regular. Small vacuoles are scattered at random throughout the cytoplasm. In the "cephalic" third of the notochord the vacuolization has progressed further. Vacuoles are enlarging, cells are consequently expanding and are not as flattened as in other parts (Fig. 11). The nucleus is polygonal in cross section, being enclosed in a larger vacuole. The diameter of the notochord equals that of the normal trunk notochord at the corresponding stage (0.11 mm.). The notochord is surrounded on all sides by the mesoderm, which shows at some points the first sign of myotome formation. Near each end of the notochord a small cavity is formed lined by a columnar epithelium with a pigmented surface. They seem to represent the neural tubes. Both tubes are at many points continuous with the adjoining mesoderm, which is developing into myotomes. Moreover, at the "caudal" end the notochord is directly continuous with the wall of the neural tube. An endoderm layer with an uneven surface covers all these structures.

Observations during the cultivation may be summarized as follows. A large number of cells were discharged just after the treatment and also during the first three days of cultivation. Not only smaller ectodermal and mesodermal cells, but also large yolk cells were found among the free discharged cells. After the recovery, the external surface of the aggregate was mostly covered by the whitish endoderm layer. The ectoderm, if present, withdrew into the interior, often leaving a small neuralized fragment on the surface, except in several cases where the epidermis covered one part of the explant. As a rule, the mesoderm occupied the interior of the explant. The form change during the subsequent cultivation was

TABLE III

Differentiation of the isolated medio-ventral marginal zone treated with ammonia

Number of explants	Frequency of rudiments and tissues differentiated in the explants									
	Notochord	Muscle	Myoblasts	Limb-bud	Nephric tubules	Mesenchyme II	Melanophores	Blood cells	Neural tissue	Otic vesicle
40	30 75%	25 62%	16 40%	2 5%	29 72%	10 25%	8 20%	2 5%	30 75%	2 5%

pronounced only in three cases where the tail developed from the explant, covered partly by the epidermis. A much less pronounced protuberance often developed on the surface covered by the endoderm. The free spherical, half-transparent body covered by the epidermis, often observed in control series II, did not occur. The explants were cultured for eight to seventeen days at room temperature before fixation in Bouin's fluid.

The results of the microscopic examination are summarized in Table III. Notochords obtained here have a varied appearance. Of thirty cases with notochordal differentiation, eight show only a small number of scattered vacuolated cells among the myotomes, while in other cases (twenty-two explants, 55 per cent of all available cases) a more or less defined notochord is differentiated. Of these cases six show an almost typical notochord with the diameter corresponding to that of a normal one. Very often (sixteen cases) the notochord occurs in an irregularly

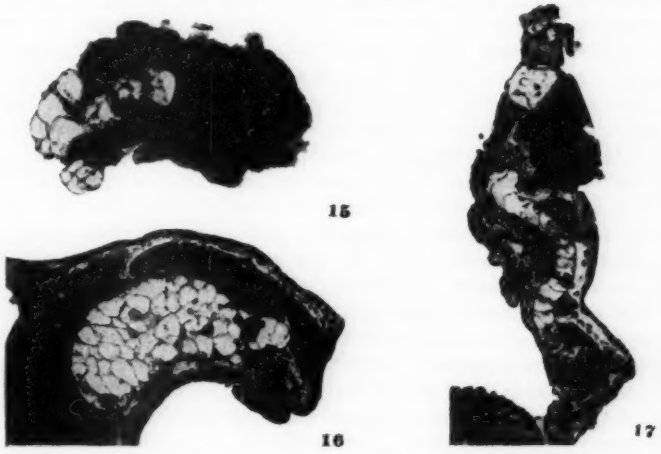


FIGURE 15. Uncovered notochord from the isolated ventral marginal zone treated with ammonia, accompanied by neural tissue and muscle cells.

FIGURE 16. A large notochord with myotome and spinal cord, induced with ammonia-treatment. The upper side of the explant is covered by the epidermis, the lower side with endoderm.

FIGURE 17. A tail-like structure developed from the explant, shown in Figure 16. A spiral notochord, with irregular mass of the myotome and neural tissue. All figures × ca. 60.

twisted form or in an aberrant form. These are found among neural tissue, myotomes or undifferentiated endoderm, exhibiting a great variation in the size of each vacuolated cell. Often cell types, which are intermediate between notochord, neural tissue and myotome, accompany these atypically formed notochords (Fig. 14). In only two cases the induced notochord developed a tail with accompanying neural tube and myotomes (Fig. 17); while in the other cases one might ascribe to the notochord the trunk character, since it possesses a larger diameter and does not show any tendency of pronounced stretching (Fig. 16). In most cases the notochord occurs within a covering tissue, but it may be exposed to the medium in the form of an unorganized tissue fragment (Fig. 15).

With a few minor exceptions the notochord is accompanied by myotomes and neural tissue. The myotomes attain a good differentiation and typical arrangement only when a typically formed notochord exists in their direct vicinity. The neural tissue and notochord have also an intimate relation. Often the fragments of notochord tissues are embedded in the neural tissue (Fig. 15) or are enclosed by a neural epithelium. Also, in the other cases, both tissues have the tendency to occur in association. Only two explants belonging to the group of imperfect notochordal differentiation are devoid of neural tissue. Here only a small number of notochord cells are scattered among the myotomes.

Neural tissue is formed very frequently (in thirty out of forty cases or 75 per cent). In twenty cases, it is differentiated on the surface of the explant without any characterization and cannot be identified with any part of the normal nervous system. The same holds for the aberrant neural tissues developed inside the explants (six cases). Only in three cases is a fragment of the spinal cord differentiated which runs along the notochord (Fig. 16). In two cases the neural cord is found as a component of the tail. Their morphogenesis is rather atypical. Differentiation of a brain cannot be ascertained in any explant. In two cases a structure developed which resembles the otic vesicle. One of these is accompanied by two ganglion-like cell groups. No nasal placode or lens is observed. Most of the neural tissues are partly or wholly laden with an exceptionally large amount of yolk granules. A remarkable consequence of this is the occurrence of clearly differentiated neurofibrils just beside the dense group of yolk granules. In younger explants the neural tissue and mesoderm have no clear limiting surface and one gets the impression that both tissues are derived from a common material.

A myotomal differentiation is found in thirty-nine of forty explants (97 per cent). Of them, twenty-five show the muscle cells with myofibrils, while other explants contain only myoblasts without fibrils. In at least five of the latter cases the explant was fixed too early for fibril differentiation to have occurred. The myofibrils exhibit the best differentiation when a normally formed notochord runs in the vicinity of the myotome. In these cases the parallelism between the course of the notochord and muscle cells is very conspicuous. Aberrant formation of the notochord is paralleled by disturbed arrangement of the muscle cells. There exists an almost perfect correlation between the occurrence of notochord and the differentiation of the myofibrils: (1) If the notochord is absent, fibrils are not differentiated (five cases); (2) In the presence of scattered notochordal cells, half of the explants differentiated fibrils (ten cases); (3) In the presence of a well defined notochord the fibrils are always differentiated (nineteen cases, excluding three prematurely fixed cases). A limb-bud is formed only in two explants. One of them shows an extensive proliferation of mesenchyme cells (type II). The nephric tubules are developed in twenty-nine out of forty cases. They are better differentiated when the explant contains notochord and muscle as well. A corresponding situation was found by the author in isolated mesoderm from the neurula. This influence of the axial organs on the development of the nephric system deserves further analysis. The blood island is found only in one case, in a restricted cavity between the endoderm and bottom of the culture dish. In no case did a cavity, lined with the mesothelium or filled with yolk-rich mesenchyme and blood vessels, develop in the explant.

Since in this series the number of the explants fused together varies considerably, it seems advisable to inquire whether the frequency of the notochord differentiation depends upon the number of fused pieces. In Table IV, the relation between the notochord differentiation and the number of original pieces is summarized. It appears that the notochord can develop in the explants from one isolate as well as in those from ten isolates. No significant difference in notochord frequency seems to exist between groups of one to three isolates.[2] It is probable

TABLE IV

Relation between the number of the original isolates fused together and the frequency of notochord differentiation

Number of isolates fused together	Number of cases	Explants with notochord differentiation	Frequency of notochord in %
1	14	10	71
2	11	8	72
3	12	9	75
4	1	1	(100)
6	1	1	(100)
10	1	1	(100)
Total	40	30	

that in explants with more than four isolates the frequency is higher, although the small number of cases does not allow any definite conclusion. On the other hand, the morphogenesis of the induced notochord may be influenced in some degree by the size of the explant. In explants from one isolate the notochord cells occur in small scattered groups without forming a cord-like tissue, while in explants from more than two isolates a more or less typically organized notochord can be developed.

Results of this series of experiments may be summarized as follows. The isolated, medio-ventral sector of the marginal zone of the earlier gastrula together with a small amount of adjacent ectoderm and endoderm, differentiates notochord, muscle and nephric tubules as well as neural tissues, if it is briefly exposed to an ammonia solution and then cultured in Holtfreter solution.

DISCUSSION OF RESULTS

An appropriate understanding of the results of control series I may be possible only when the behavior of the untreated explant of the ectoderm of a gastrula of *Triturus pyrrhogaster* in Holtfreter solution is known. Such explants differentiate into nothing but atypical epidermis. This result, confirmed repeatedly by the author and described in another paper in preparation, shows clearly that the isolated ectoderm of this species behaves like that of *Triturus torosus* and does not respond to isolation and cultivation by undergoing neural differentiation as does the isolated

[2] The significance of the difference in frequency of the notochord in the explants from one isolate and in the explants from three isolates in the above table, was tested after the exact treatment of R. A. Fisher and the probability of occurrence of the observed set of frequency was found to be 0.795. Hence we *cannot* conclude from the above data that notochord occurs in the explants from three isolates more frequently than in the explants from one isolate.

ectoderm of *Amblystoma punctatum*. Hence the neural differentiation obtained in control series I must be ascribed to the ammonia treatment. Study of the induced structures in this series has shown that they have more or less distinct characteristics of cephalic ectodermal rudiments and tissues such as brain, eye, nasal placode, lens, mesenchyme, melanophores and frontal gland. In another paper will be presented data which show that comparable differentiations occur in the isolated ectoderm induced by a multitude of substances and also by a living cephalic organizer; the similarities between these induced structures and corresponding normal structures cannot be a mere coincidence. The conclusions conform in general with those obtained by Holtfreter (1944a, 1945, 1947) in so far as the "self-induced" explants of *Amblystoma punctatum* and the imperfectly cytolyzed explants of *Triturus torosus* are concerned.

Comparing the results obtained in control series II and the experimental series it seems likely that the ammonia-treatment induces in the isolated medio-ventral marginal zone the differentiation of notochord and muscle, and suppresses the development of blood cells and mesothelium. But before this can be concluded we must exclude two possible sources of error: (1) *The development of dorsal tissues may be caused by the presence of some cells of the adjacent marginal zone taken along erroneously at the operation.* This possibility seems to be excluded, because in control series II it was shown that even the latero-ventral section never gave rise to notochord and muscle. It may be remembered that the dorsal limiting line of this section lies 10° more dorsal than that of the medio-ventral section, which was exclusively isolated in the experimental series (Fig. 4). (2) *The differentiation of the dorsal tissues may be brought about by the volume increase following the reaggregation of cells from many isolates into a single aggregate.* We have shown above that axial differentiation is possible even in the ammonia-treated explant originating from one isolate and having a smaller quantity than the average explant of control series II. On the other hand, in control series II, typical differentiation of the ventral mesoderm occurred even in the explants originating from six pieces of medio-ventral section. The second possible source of error can also be rejected.

Thus, it may be concluded that through ammonia-treatment the mode of differentiation of the isolated medio-ventral marginal zone can be altered, so that ventral mesodermal differentiations such as the blood island, blood vessels and mesothelium are suppressed, whereas dorsal differentiations such as the notochord and muscle are initiated, and intermediate differentiations such as the nephric tubules are not significantly altered (Table V).

The close parallelism and intimate spatial relation between the notochord and neural tissues observed in the experimental series deserves special attention. This correlation can be most readily explained if we assume that the neural tissue is induced in the ectodermal component of the explant by that part of the mesoderm which later differentiates into notochord and muscle. However, it must be recognized that the following possibilities are by no means excluded: (a) The neural differentiation and notochordal differentiation are induced separately in the ectodermal and mesodermal component respectively by the ammonia-treatment. The observed spatial relation between both tissues results from a later mutual approach and fusion. That such mutual approach occurs between tissues of the same nature is abundantly exemplified (Yamada, 1937; Holtfreter, 1944b) and it is not improbable that an analogous attraction also exists between different tissues of the

TABLE V

Comparison of the mode of differentiation of the ammonia-treated and non-treated isolate from the ventral marginal zone

Differentiation	Frequency of tissues in %	
	Control series II (non-treated)	Experimental series (ammonia-treated)
Notochord	0	75%
Muscle	0	62%
Mesenchyme II	5%	25%
Melanophores	11%	20%
Nephric tubules	56%	72%
Blood cells	70%	5%
Blood vessels	20%	0
Mesothelium	21%	0

axial region. (b) One part of the neural tissue obtained did not originate from the ectodermal component but from that part of the mesoderm which otherwise became notochord and muscle. Holtfreter (1938) showed that the dorsal marginal zone has the tendency to differentiate neural and other ectodermal tissues, besides notochord and mesodermal tissues, if it is isolated and cultured in Holtfreter solution. If, in the present case, ammonia-treatment makes the ventral marginal zone equivalent to the dorsal marginal zone, it is very probable that the same process can occur in these explants. Indeed, the morphogenesis of the ammonia-treated isolate of the ventral marginal zone does not differ much from that of the isolated organizer. As repeatedly pointed out above, a part of the neural tissue obtained in the explant shows intimate connections with the mesoderm and often a very high content of yolk granules. These facts can be most readily explained if we assume these possibilities: (c) One part of the obtained notochord did not originate from the mesodermal component but from the ectodermal component of the isolate, which was otherwise neuralized. This is not impossible since the presumptive ectoderm can be induced to form notochord and muscle, if it is applied to the trunk mesoderm of the neurula (Holtfreter, 1933). (d) The differentiation of the notochord was evoked in the mesoderm by the presence of the ectoderm which was neuralized by the treatment. This possibility might seem to contradict the observed fact that the neuralized tissue forms (in control series I) rudiments of more or less distinctive cephalic type, while in the experimental series the neuralized tissue shows characteristics of trunk and tail. This fact apparently supports the first assumption that the neural tissue is induced secondarily by the notochord and mesoderm. In spite of this we must not completely neglect the possibility that the neuralized ectoderm acts as an inducer on the mesoderm, while the subsequent differentiation of the ectoderm is inversely governed by the mesoderm or notochord and, consequently, the neural tissue acquires a trunk or tail nature (Yamada, 1939).

It is probable that many of these possibilities are realized in our experiments. An experimental test of these possibilities is not as simple as one might expect, as the boundary between the ectoderm and mesoderm is very indistinct on the surface of the early gastrula, and some observations suggest that the induced differentiation of the notochord is connected with this boundary. This problem will be the subject of a further series of experiments.

Theoretical Considerations

In order to discuss the theoretical significance of the present results, it seems necessary to introduce the double-potential theory proposed by the writer in an article written in Japanese (Yamada, 1947), as it furnishes the basis for the present investigation.

The essential points of the theory can be described most concisely as follows: (1) The developmental activity of a given germ region depends, above all, upon two sorts of "potentials," [3] which are designated respectively the dorso-ventral and cephalo-caudal, and can vary relatively independently of each other. (2) At a given developmental stage, the dorso-ventral and cephalo-caudal potentials show a definite gradient along the respective axis; or, otherwise expressed, every germ region has a definite value of both potentials according to its topographical position relative to both axes. (3) In the course of development the absolute values of both potentials show a continual change, which is characteristic for the germ region and for the stage. Thus the developmental activity of any germ region may be defined as the combined effects of the time-curves of both potentials. (4) The change in potential is effected partly through the factors inherent in the germ region and partly through factors external to it. The latter factors are called the mediators. There are two of them . . . the dorso-ventral and cephalo-caudal mediators affecting respectively the dorso-ventral and cephalo-caudal potentials.

This theoretical scheme is constructed to deal with the early ontogenesis of amphibians, but it is expected that the same principle can be adapted to other developmental systems, with alterations of unessential points.

To apply this scheme to the present problem, we have to follow some suggestions made earlier: (a) Considering the analysis of the normal course of development and the experimental data, it was assumed that the dorso-ventral potential is connected primarily with the intensity of the biochemical activity of the developing system, while the cephalo-caudal potential is connected primarily with the morphogenetic movements of the developing system. (b) Most of the phenomena, called embryonic induction, involve a shift of the dorso-ventral potential toward the dorsal end, and may be in this sense called dorsalization. The dorso-ventral mediator, which is responsible for this effect, seems to be (in most cases) chemical in nature. Many chemical substances or procedures can play the role of a dorso-ventral mediator. (c) The morphogenetic indifferent state, such as is found in the presumptive ectoderm of the early gastrula of many amphibians, is characterized by the ventral (low) value of the dorso-ventral potential and the cephalic (low) value of the cephalo-caudal potential. This means that through the action of any dorso-ventral mediator, such a germ region can be induced to form cephalic axial organs (eye, brain and nose), while without such action of mediators this germ region will differentiate only epidermis in the urodeles and epidermis and sucker (the cephalic and ventral differentiation) in the anurans. If we want to get caudal axial organs out of the same material, the cephalo-caudal mediator must be applied as well as the dorso-ventral mediator, to shift the cephalo-caudal potential in caudal direction. The ventral value

[3] The concept of "morphogenetic potential" was first introduced by Dalcq and Pasteels (1937) in their theory of morphogenesis. As will be clear from the following text, in our theory a meaning is given to this concept which differs much from that in the theory of the Belgian authors. However their basic contributions to the theoretical problem here treated must be appreciated.

of the dorso-ventral potential and the cephalic value of the cephalo-caudal potential being called "low," the effect of the dorso-ventral and cephalo-caudal mediator can be designated, in this case, as a "rise" in respective potential value. (d) If a germ region with a high value of one of the potentials comes in contact with a germ region with a low value of the same potential, the former generally acts as the mediator, provided that an appropriate contact surface is established. The organizer acts through the coordinated spatio-temporal pattern of both mediators, which is found in the potential composition of the germ region concerned, i.e., the dorsal marginal zone.

From the standpoint of the double-potential theory the morphogenetic effect of sublethal cytolysis can be designated as dorsalization; in other words it raises the dorso-ventral potential of the developing system. If the presumptive ectoderm of the early gastrula, with low values of both dorso-ventral potential (*Pdv*) and cephalo-caudal potential (*Pcc*), is treated with a sublethal dose of the cytolyzing agent, the *Pdv* will be raised to a higher value while the *Pcc* will be left unchanged. The end result must be a dorsal cephalic differentiation (such as brain and eye) without additional caudal differentiations (ear vesicle, spinal cord, notochord and muscle). The facts correspond well to this expectation. One of the most striking and, until now, unexplained features of the "self-induced" ectoderm is that it comprises almost exclusively structures of the foremost region of the head (Holtfreter, 1948) and is thus just what is to be expected from our theory.[4] The qualitative difference within the obtained cephalic inductions may be attributed, by this theory, to the difference of *Pdv*-curve and its spatial distribution in each case. A sharp rise of the *Pdv*-curve leading to the maximal value may be followed by differentiation of the eye. A less pronounced elevation may lead to the brain. A still less pronounced one corresponds to the nose-type, and a weak one, perhaps, to the mesenchyme and chromatophores. A rise of *Pdv*, in a small circumscribed area, may cause the differentiation of the lentoid.

It must be admitted that such a presentation neglects the normal elaboration of the organ rudiments. If we distinguish two phases in the development of an organ-rudiment from the germ layer, *viz.*, the phase of determination of the basic pattern of the organ, and the phase of elaboration of this pattern, the above discussion deals only with the former. For the latter we must perhaps take other factors into account, which need not be specific. In the isolate from earlier developmental stages the phase of elaboration seems to be absent, and the structures developed within it are, in no case, normally elaborated. As this imperfection of elaboration occurs also in the isolate with the living organizer, we cannot ascribe it wholly to an inadequate nature of the primary inductor.

What, therefore, will be expected from the theory when the same treatment is applied to the ventral marginal zone of the early gastrula? This germ region can be characterized as having a low *Pdv* and an intermediate *Pcc*, or more precisely, as being endowed with the inherent capacity to raise its *Pcc* to an intermediate level, because it gives rise to the ventral differentiations of the trunk region in normal development and also in isolation (control series II). The ammonia-treatment must, in this case, change only *Pdv* leaving the *Pcc* unaffected. The results may be the maximal value of *Pdv* and an intermediate value of *Pcc*. This means the

[4] It may be added in this connection that the theory was proposed at the time when the works of Holtfreter (1944, 1945 and 1947) were unknown in Japan.

differentiation of the axial organs of the trunk. Our results (experimental series) fulfill the theoretical expectation. The regional differences of *Pdv* within the isolated mesoderm may account for the occurrence of different tissues side by side; the maximal value corresponding to the notochord, the next highest value to the muscle and the intermediate one to the pronephros. That a good differentiation of the myofibrils occurs only in the presence of a notochord may be interpreted by the assumption that this differentiation presupposes a gradual rise of *Pdv* in the later stages, caused by the notochord which plays the role of dorso-ventral mediator owing to its special histogenesis. But we must not neglect the possibility that stretching of the muscle, which is caused by the notochord, plays an important role in the formation of its fibrils.

It is well known that many physiological and histochemical reactions show clear dorso-ventral gradients which are superimposable upon each other. This type of gradient is, for instance, exhibited by cytochrome oxidase, sulfhydryl groups of protein, ribonucleic acid, alkaline phosphatase and acetalphosphatide. Recent studies make it probable that at least some of these reactions are bound to submicroscopic granules called microsomes (Brachet, 1949). Studying the effect of cytolysis on the behavior of cytoplasmic ribonucleic acid, J. Brachet (1946) finds that the basophilia of the cytoplasm of embryonic cells of amphibia markedly increase when exposed to an abnormal salt solution, and that this basophilic material can be digested away by a dilute solution of crystalline ribonuclease. Holtfreter (1948) reports that an exposure of isolated embryonic tissue to salt solution of high pH produces "appearance and precipitation of basophilic granules in the previously hyaline portion of the protoplasm." From these observations it seems probable that ammonia-treatment causes an increase of the basophilic granules in the ectoderm as well as in the mesoderm. If this is the case, then there exists a clear parallel between the concentration of basophilic granules and *Pdv* in experimental as well as in normal conditions. In both cases the dorsal mode of development occurs in the region with high granular content, and the ventral mode in the region with low content. This may mean that *Pdv* is nothing but the capacity of cells to maintain or produce a certain concentration of these granules. Recent data on the role of ribonucleic acid in many cytological elaborations and formation of fiber protein (Brachet, 1949), make it probable that differentiations of neurofibrils, myofibrils and notochord vacuoles (which occur exclusively in the region of high *Pdv*) are in some way connected with the basophilic granules.

Summary

(1) Control series I. The ectoderm of the early gastrula of *Triturus pyrrhogaster* was isolated and exposed for a short time to a solution of ammonia and then cultured in Holtfreter solution. The isolate differentiated structures which can be characterized as cephalic ectodermal rudiments including brain, eye, nose and frontal glands. Otic vesicles, spinal cord and mesodermal structures were wholly absent.

(2) Control series II. The ventral marginal zone of the early gastrula was isolated and cultured in Holtfreter solution. It differentiated blood island, blood vessels and mesothelium and nephric tubules.

(3) Experimental series. The isolated ventral marginal zone was treated with ammonia solution and cultured in Holtfreter solution. It gave rise to notochord

and muscle as well as nephric tubules. The development of the blood island, mesothelium and blood vessels was almost completely suppressed.

(4) Results were interpreted from the standpoint of the double-potential theory (Yamada, 1947).

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SEROLOGICAL SYSTEMATICS OF SOME PALINURAN AND ASTACURAN CRUSTACEA[1]

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Introduction

Only a few serological tests indicating the taxonomic relationships among the long-tailed Crustacea are recorded in the literature. Nuttall (1904) and Graham-Smith (1904), both using the same antisera made against *Homarus vulgaris* and *Potamobius astacus,* separately tested these antisera with a large variety of animal sera; of these antigens only those of the decapod Crustacea gave positive reactions. The results obtained by these authors, in some instances, were not in accord with accepted zoological classifications. The serum of *Palinurus vulgaris,* for example, reacted more weakly with the anti-*Homarus vulgaris* serum than did the sera of certain brachyuran species. Erhardt (1929) produced an antiserum against the serum of the crayfish *Potamobius astacus* and tested it with the homologous serum, plus the sera of *Potamobius leptodactylus,* the lobster *Homarus vulgaris,* several brachyuran species, and other invertebrates. Except for an unexplained heterologous reaction with the serum of the snail, *Helix pomatia,* only the crayfishes and lobster reacted, and these in accordance with the taxonomic positions previously assigned to them on the basis of information obtained from non-serological data. That the sera used as antigens by these early workers were not standardized in terms of protein content may, in part, explain the unexpected results sometimes obtained when the reactivity of their apparently non-comparable antigen dilutions were compared.

Clark and Burnet (1942) using the ring test sensitivity method, and unstandardized antigen dilution series, were able to separate various genera of the Parastacidae from each other and from other Decapods.

In 1943, Boyden, using a turbidimetric analysis of the whole precipitin range, measured the degree of serological correspondence between the sera of *Homarus americanus* and *Homarus vulgaris* with an appropriate antiserum. The index of serological activity used by this author was a value proportionate to the whole curve area. This was the first application of a quantitative technique to obtain serological data that was to be used in judging the taxonomic relationships of the long-tailed Crustacea.

The present writer made a preliminary report (Leone, In press) of his investigations of the sera of invertebrates, and included in it some of the results reported below. The object of this present report is to record further serological data that

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will aid in making a more accurate taxonomic arrangement of the palinuran and astacuran Crustacea.

Materials and Methods

As a result of the establishment of the Serological Museum of Rutgers University (Boyden, 1948), cooperating institutions and individuals from several parts of the world have aided in collecting and providing the samples of animal sera used in this investigation. Sera of species of the Australian genera *Euastacus* and *Cherax* were obtained through the efforts of Miss Ellen Clark of Victoria. Sera of the Hawaiian species of *Panulirus* were provided by Dr. Bradley T. Scheer of the University of Hawaii. Sera of the American west-coast spiny lobster were provided by Dr. Albert Tyler, California Institute of Technology, Pasadena, California. The other samples of sera came from stocks in the Serological Museum, which were collected chiefly by Dr. Alan A. Boyden, Rutgers University. Table I contains a list of all the antigens used and the antisera prepared.

TABLE I

A list of the species, and their sources, whose sera were used in making the serological comparisons reported upon in this paper

Species	Sample	Source	Antiserum
Panulirus argus (Latreille)	39–1 ♂	Tortugas, Florida, U.S.A.	I–76
Panulirus argus (Latreille)	39–3 ♂	Tortugas, Florida, U.S.A.	I–77
Panulirus interruptus (Randall)	46–1	California Institute of Technology, California, U.S.A.	I–56
Panulirus penicillatus (Oliver)	49–1	Honolulu, Hawaii	I–117
Panulirus japonicus (de Siebold)	49–1	Honolulu, Hawaii	
Palinurus vulgaris Latreille	No. 1	Plymouth, England	I–93
Homarus vulgaris Milne-Edwards	No. 1	Plymouth, England	I–91
Homarus americanus Milne-Edwards	36–2d	Mt. Desert Island, Maine, U.S.A.	I–89
Cambarus clarkii Girard	36–1	(Unknown)	I–92
Euastacus elongatus Clark	48–1	Victoria, Australia	I–116
Euastacus nobilis(Dana)	48–1	Victoria, Australia	
Euastacus armatus (von Martens)	48–1	Victoria, Australia	
Cherax destructor Clark	48–1	Victoria, Australia	
Cherax albidus Clark	48–1	Victoria, Australia	

The details of the technique of testing have been considered elsewhere (Boyden, 1942; Boyden and DeFalco, 1943; Leone, 1949). Briefly, the method employed is to measure, by means of a photoelectric turbidimeter, the precipitate developed in an antigen dilution series, after a constant amount of antiserum has been added to each dilution. The range of antigen concentrations extends from complete antigen excess to complete antiserum excess. Also considered in these earlier papers were various problems related to establishing the validity of the results of tests made with sera collected over a period of years, and which were processed in various ways. In general, the serum antigens proved to be representative of the organisms from which they came, and were suitable reagents with which to conduct researches in serological systematics. The customary twenty-minute reaction time at 38° C. was employed in these tests. In examining the degree of serological correspond-

ence [3] among the sera of the various species in the genera *Panulirus* and *Euastacus* a twenty-four hour reaction time at room temperature was employed, as well as the short time readings.

Experimental Results

The results of the serological tests are summarized in Table II and Table III.

Table II

Serological Relationships

Antigens	Antisera							
	Panulirus argus	*Panulirus interruptus*	*Panulirus penicillatus*	*Palinurus vulgaris*	*Homarus americanus*	*Homarus vulgaris*	*Cambarus clarkii*	*Euastacus elongatus*
Panulirus argus	100	86	88	35	5	5	6	4
Panulirus interruptus	85	100	88	31	5	5	5	4
Panulirus penicillatus	88	88	100	33	4	4	4	4
Panulirus japonicus	70	71	77		13		4	
Palinurus vulgaris	54	31	50	100	4	7	5	3
Homarus americanus	14	9	15	5	100	96	50	10
Homarus vulgaris	15	11	16	6	88	100	48	10
Cambarus clarkii	13	11	14	6	50	48	100	38
Euastacus elongatus	13	11	14	6	54	49	57	100
Euastacus nobilis	13	10	14	7	53	49	58	89
Euastacus armatus	13	11	13	7	53	47	55	88
Cherax destructor	12	9	11	6	47	47	65	61
Cherax albidus	10	8	12	6	48	45	63	58

The numbers in the table are percentages of the value of the homologous reaction which is designated as 100 per cent. Values within 5 per cent of one another are generally considered to be within the experimental error of the method. All values are averaged from two or more tests, except those for *Panulirus japonicus*. It is readily seen that the various antisera differ in their capacity to react with the heterologous sera. All values are for 20 minute reaction time.

Of the species tested in the genus *Panulirus*, a high degree of correspondence is shown by the sera of the spiny lobsters, *P. argus*, *P. interruptus*, and *P. penicillatus*. On the basis of the serological evidence alone, these three species are in the same category, or are in closely related categories, whereas *P. japonicum* is in another category.[4] The European spiny lobster, *Palinurus vulgaris*, is in still a different category and the degree of difference between it and the others is so great as to suggest differences on the generic level.

[3] Serological correspondence. As applied to antigens it includes all antigens capable of reacting with an antiserum to any one of them. As applied to antibodies, or antisera, it includes all antibodies or antisera capable of reacting with a given antigen or hapten. Whenever the serological correspondence is exact we may speak of serological equivalence (Boyden, 1942).

[4] The serum sample of this species had a soluble protein concentration of less than one per cent, and, in addition, was preserved in merthiolate colored with eosin. Consequently the contents of the tubes in the antigen excess region of the reaction curve were dark red. The amount of reaction recorded seemed to be less than a visual estimate of the dilution tubes would indicate. The antiserum excess region of the curve was normal. Any change, applied as a correction, would be in the direction of a closer correspondence to the homologous antigen indicating less difference between *P. japonicum* and the other palinuran species than is indicated in the table.

TABLE III

A comparison of 20 minute and 24 hour reaction times

Species	Reaction time					
	20′	24 hr.	20′	24 hr.	20′	24 hr.
Panulirus penicillatus	100	100	88	100	86	96
Panulirus argus	88	102	100	100	85	99
Panulirus interruptus	87	100	88	99	100	100
Euastacus elongatus	100	100				
Euastacus armatus	88	98				
Euastacus nobilis	89	96				

Values indicate the per cent relationship to the homologous reaction. The antigenic correspondence indicated after 20 minutes is high; after 24 hours correspondence in antigen reaction is exact.

The true lobsters, *Homarus americanus* and *Homarus vulgaris,* also show a high degree of serological correspondence. Whether or not the difference between them is sufficient to merit separate specific status for each is open to question from the serological point of view.

The three species of the genus *Euastacus* closely correspond to one another serologically, the heterologous reactions being only 11 to 12 per cent less than the homologous reaction. As it was in the cases of the palinuran organisms, these astacuran species show less than the 30–50 per cent difference from the homologous reaction which has been suggested (Boyden, 1943) as defining species in serological tests involving decapod Crustacea. As Boyden pointed out (personal communication), these values depend upon the grade of specificity of the antisera used and have reference to comparable antisera only.

The serum of *Cambarus clarkii* is notably different from the sera of the other astacuran species. The sera of species of the fresh water genera *Euastacus* and *Cherax* react more strongly with the anti-*Cambarus* serum than do the sera of the true lobsters of the genus *Homarus.* The sera of *Homarus,* on the other hand, react considerably more with anti-*Cambarus* serum than do the sera of any of the palinuran, spiny lobsters. Sera of species of the genus *Cherax* gave slightly greater reactions with the anti-*Euastacus* serum than did the serum of *Cambarus.*

Various species of brachyuran Crustacea were tested with each of the antisera shown in Table I. Heterologous reactions were obtained in some tests, indicating serological correspondence. In no test, however, did the serum reaction of any brachyuran exceed, or approach for that matter, the weakest heterologous reaction of the palinuran and astacuran species.

In order to ascertain whether the differences in the reactivity of heterologous antigens of high serological correspondence were constant, some of the values for twenty-minute reaction times were compared with twenty-four hour reaction times. A period of twenty-four hours at room temperatures was sufficient time for the precipitin reactions to go to completion. Merthiolate employed at a concentration of 1 : 10,000 in the saline diluent was adequate to inhibit bacterial growth during the test period.

From the data in Table III it can be seen that the heterologous test values which differ from the homologous by as much as fifteen per cent after twenty-minutes reaction time, may appear to be serologically equivalent after twenty-four hours of reaction time. Reactions with values within five per cent of the homologous value are considered equivalent. The sera of the three listed species of the genus *Panulirus* appear to be serologically equivalent, after twenty-four hours, as are the sera of the three species of the genus *Euastacus*. When the correspondence of the sera of heterologous species is as high as it was in these tests, experience has taught us that detectable differences, as revealed by other criteria, are slight. Consequently the usefulness of serum as a precise taxonomic tool is demonstrated. These observations are pertinent for the sera of Crustacea which possess only a single kind of antigenic protein, the hemocyanins (Allison and Cole, 1940; Clark and Burnet, 1942; Tyler and Scheer, 1945), and are, by their nature, relatively pure serological reagents. Serological reactions with these sera are not so subject to the inconsistencies and irregularities which sometimes are revealed, with proper techniques, in mixed antigens such as the sera of mammals which contain several antigenically distinct proteins (Serological Museum, Bulletin No. 2, 1948).

Summary

From the serological studies of the palinuran and astacuran Crustacea it is concluded that:

1. Sera of species within a genus react more strongly with an antiserum made against one of them than with any other antiserum.

2. Sera of different species within the same genus may show different degrees of relationship to an antiserum made against the serum of one member of that genus.

3. The sera of representatives of different genera in the same family show different degrees of relationship to one another.

4. The sera of species within a family react to a greater extent with antisera made against the sera of members of that family than with any other antisera.

5. *Panulirus argus, Panulirus interruptus,* and *Panulirus penicillatus* correspond almost exactly with one another serologically, and on the basis of the serological evidence presented, may be considered the same, or closely related species. This is in spite of their wide separation geographically.

6. *Euastacus elongatus, Euastacus nobilis,* and *Euastacus armatus,* serologically, closely correspond with one another. Probably they are the same, or closely related, species.

7. In the genus *Homarus,* the two species *H. americanus* and *H. vulgaris* closely correspond with each other, and probably are the same, or closely related species.

8. Differences in per cent of serological relationship among crustacean sera tested with the same antiserum are attributable to differences in their hemocyanins.

9. Twenty-minute reaction times for precipitin reactions establish significant orders of relationship among animal sera. Twenty-four hours reaction time does not alter the order of relationship from that established at twenty minutes despite considerable increases in amounts of reaction recorded.

10. Differences detectable among antigens of high correspondence, after a twenty-minute reaction time, may disappear after a reaction time of twenty-four hours.

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LYTIC EFFECTS OF SPERM EXTRACTS ON THE EGGS OF MYTILUS EDULIS[1]

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INTRODUCTION

A variety of specific substances are obtainable from the eggs and sperm of animals (reviewed by Tyler, 1948, and Bielig and von Medem, 1949). From the sperm may be obtained antifertilizins and various lytic substances such as hyaluronidase from mammalian sperm, an egg surface lysin from sea-urchin sperm, and membrane lysins from the sperm of various animals.

As described in a preliminary note (Berg, 1949) sperm extracts of *Mytilus edulis* are capable of dissolving or preventing the formation of the cement which normally binds the segmentation blastomeres together. The present report concerns the characteristics of the active agent particularly with respect to its chemical nature and relationship to an egg membrane lysin which is also present in the sperm extracts. The cement dissolving activity of the extracts is closely associated with the membrane lysin; however there is evidence which suggests that the effect is brought about by a separate substance.

Lysis of jelly coats and membranes of eggs by sperm extracts has been described by previous investigators. Hibbard (1928) and Wintrebert (1929, 1933) discovered that strong sperm suspensions or sperm extracts of the amphibian, *Discoglossus*, caused dissolution of an egg coat. Tyler (1939) extracted membrane lysins from the sperm of the keyhole limpet, *Megathura crenulata*, and the abalone, *Haliotis crackerodii*, which dissolved the membranes of the eggs. von Medem (1942, 1945) subsequently described similar membrane lysins in other species of limpets and abalones. Ruffo and Monroy (1947) reported the dissolution of the egg coats of several species of sea-urchins by a hyaluronidase-like substance obtainable from the sperm, and Monroy (1948) later reported the presence of a membrane lysin in the sperm of *Pomatoceros*.

The action of the above membrane lysins on cleavage of the eggs was in some instances not reported or claimed to have no effect.

METHODS

The gametes of the bay mussel, *Mytilus edulis*, were used for the majority of these experiments, although at times the testes of the larger species, *Mytilus californianus*, were used for preparing considerable quantities of sperm extracts. Animals, collected from the San Francisco Bay or the open coast, were kept at 5° C.

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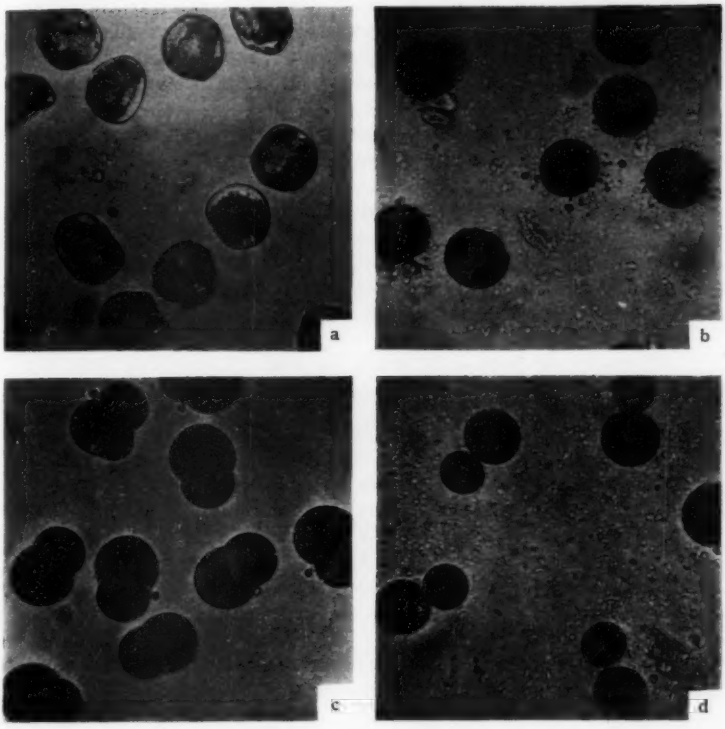
PLATE 1



Effects of *Mytilus edulis* sperm extracts on the egg membrane and intercellular cement: a, membranes rendered visible by plasmolysis of eggs with hypertonic sea water; b, dissolution of membranes after two minutes treatment with a sperm extract; c, normal appearance of eggs after first cleavage; d, cleavage of eggs in a sperm extract.

for as long as three or four days. Viable eggs and sperm were obtained by allowing the animals to spawn in individual finger bowls of sea water at room temperature.

Sperm suspensions, used for the preparation of sperm extracts, were obtained either by concentrating naturally spawned sperm by centrifugation or by the shedding of sperm from a number of excised testes in a small volume of sea water. The latter procedure proved to be particularly convenient for obtaining very dense suspensions of sperm. Sperm extracts were prepared by freezing a sperm suspension followed by thawing and grinding the frozen mass in a mortar. This resulted in a viscous liquid which on centrifugation separated into a white precipitate and a straw colored supernatant. The latter, representing a crude sperm extract, contained the egg membrane lysin and cement dissolving factor.

Ripe eggs of *M. edulis*, as obtained by spawning, are about 63 microns in diameter, somewhat opaque, and irregularly shaped. There is no egg coat or jelly layer

although a thin membrane can be made visible by cytolysis of the egg in distilled water or by plasmolysis in hypertonic sea water. The latter treatment was used as a standard method for revealing the membranes for subsequent tests in sperm extracts. Sea water concentrated to nearly twice the normal strength was used as a plasmolytic agent, and after 10–20 minutes exposure to this solution the membranes became clearly visible (Plate 1a).

The cement dissolving activity of a sperm extract was determined by whether the first cleavage blastomeres became noncohesive and spherical. Since the membranes themselves act as physical hindrances to separation or rounding up of the blastomeres, they were routinely removed from the eggs shortly after fertilization. As a standard procedure the membranes were removed by a strong sperm extract 10–20 minutes after fertilization of the eggs. The "naked" eggs were transferred through several dishes of sea water to remove all traces of the sperm extract and then added to the test sperm extracts 10–15 minutes before the first cleavage.

Effects of Sperm Extract on Egg Membranes and Intercellular Cement

The presence of an egg membrane lysin in *M. edulis* sperm extracts is easily demonstrated. An extract prepared by the method of freezing and thawing will cause the disintegration of the membranes in a few seconds or minutes depending upon its strength. The dissolution begins by a thinning and buckling of the membrane. Gradually the sharp edges become less distinct until only a residue remains as a corona about the egg surface. In all except weak sperm extracts, this corona eventually disappears. Plate 1a shows the appearance of the membranes after plasmolysis of the eggs and 1b shows eggs of the same sample two minutes after treatment with a sperm extract.

The preceding plasmolytic treatment necessary to expose the membranes has no effect on the lytic process since the membranes can be dissolved as readily from unplasmolyzed eggs.

Sperm extracts, in addition to dissolving the membranes, also have a striking effect on the early cleavages of the eggs. Cleavage of *M. edulis* eggs has been described and pictured in detail by Field (1922) and only a brief account need be given here. Shortly after fertilization the eggs lose their irregular shapes and by the time the first polar body is formed they are spherical. Shortly after the second polar body is formed the egg surface in the vegetal hemisphere becomes wrinkled, followed by a bulging of the cytoplasm in this area. The cytoplasmic bulge, or polar lobe, causes the egg to assume a pear shape (Fig. 1a) and as the lobe increases in size the egg becomes flattened and furrowed in the animal hemisphere (Fig. 1b). This gives the illusion that the egg has divided into three cells, the so-called trefoil stage. The first cleavage plane, beginning at the animal pole, curves to one side in the vegetal hemisphere, so that the polar lobe is separated from the AB cell, but retains connection with the CD cell (Fig. 1b). Fusion of the polar lobe with the CD cell occurs soon after the trefoil stage resulting finally in an unequal cleavage. The first cleavage blastomeres are strongly cohesive and there is a considerable flattening of their area of contact (Fig. 1c, Plate 1c).

If eggs are allowed to cleave in a sperm extract, the appearance of the polar lobe and blastomeres is quite different from the normal pattern. The polar lobe appears

as a bulge in the vegetal hemisphere, as in the controls, and the eggs assume a pear shape (Fig. 1d). However, shortly afterwards, at what would correspond to the trefoil stage, the appearance diverges from that of the controls. At this stage (Fig. 1e) the polar lobe extends directly out from the eggs as a globule of cytoplasm connected to the CD cell by a stalk. The plane of cleavage is straight instead of curved and as the furrow advances across the cytoplasm the newly formed edges become separated instead of cohering as in the controls. After cleavage the polar lobe is retracted into the CD cell, although occasionally it may be pinched off completely. At the close of first cleavage, the blastomeres are spherical and may be either completely separated or barely touching (Fig. 1f, Plate 1d). In a weak sperm extract the blastomeres remain attached, but the area of contact is noticeably reduced as compared to the controls.

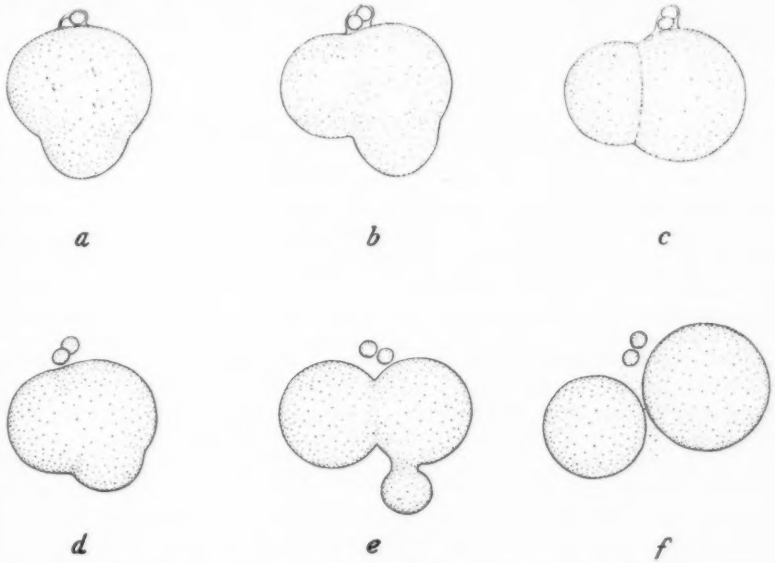


FIGURE 1. Normal first cleavage of *Mytilus edulis* eggs (a-c) and cleavage in a sperm extract (d-f) : a, formation of polar lobe; b, trefoil stage; c, completion of first cleavage; d-f, corresponding stages of cleavage in a sperm extract.

The cement dissolving activity of sperm extracts is maximal during the process of cleavage. Eggs which have already completed the first cleavage are affected very little. However, if they are allowed to remain in the extract, the A, B, C and D cells, formed by the second cleavage, become noncoherent and assume a spherical shape. Embryos which are reared in sperm extracts usually develop into a loose mass of ciliated cells.

Essentially the same pattern of cleavage and development is obtained if the eggs are allowed to cleave in calcium free sea water. Although there are slight differences in appearance of eggs cleaving in a sperm extract as compared to those in calcium free sea water, it seems reasonable to assume that the sperm extract acts

in a similar manner as lack of calcium—by dissolving or preventing the formation of the cement which holds the cells together.

Fertilized eggs which have had the membranes removed, often become sticky and adhere to the bottom of the containers. Addition of a sperm extract causes the adhesiveness to disappear which supports the view that the extract contains a substance capable of dissolving the cement. This, in the broad sense of the term, may be considered a lytic activity of the sperm extracts and the active agent will be referred to tentatively as the cement lysin.

Properties and Relationship of Active Agents in the Sperm Extracts

A lytic action of sperm extracts on the intercellular cement has not been described before, either in *Mytilus* or other forms, and it seemed worth-while to investigate the causative agent in regard to its occurrence, identity or non-identity with the membrane lysin, and some of its chemical properties.

Table 1

Bioassays of the lytic activities of various types of sperm extracts.
Each figure represents a separate experiment.

	Time for membrane dissolution of minutes	Titer of cement lysin
Concentrated sperm suspensions	4, 6	16, 32
Supernatants of above suspensions	Slight effect after 45 min. to 1 hour	4, 8, 8
Extraction by freezing and thawing	2, 5, 3	128, 64, 64
Extraction by heat	No effect	16, 16
Acid extraction	5, 5	32, 64
Alkaline extraction	15	16

In many of the experiments to be described, it was necessary to use bioassays of the sperm extracts. The time interval for dissolution of the egg membrane is a satisfactory assay for the membrane lysin; however, there is no such convenient time factor involved in the dissolution of the cement. Accordingly a dilution assay was employed in which the sperm extract was diluted ½, ¼, ⅛, etc., and the greatest dilution at which a perceptible effect occurred indicated the strength of the original sperm extract. The strength or titer of the extract was most conveniently expressed as the reciprocal of the greatest dilution exhibiting an effect. A strong sperm extract may have a cement dissolving titer as high as 128; accordingly there is a considerable range for comparison of different extracts. Using these bioassays, the strengths of the effective agents were determined in sperm suspensions, their supernatants, and in extracts prepared by various methods. Results of typical experiments are summarized in Table 1.

Dilute sperm suspensions (as obtained by spawning) and their supernatants had no perceptible effect on the egg membranes or early cleavages. Sperm suspensions, roughly about fifty times as concentrated as above, were prepared by the shedding of the sperm from excised testes. These suspensions dissolved the membranes about as rapidly as extracts prepared from the same suspensions, but the cement dissolving titers were proportionately weak as compared to the extracts (Table 1). This difference, however, seems too slight to be interpreted as any real separation of lytic activities. The supernatants, as obtained by centrifugation, caused dissolution of the intercellular cement, but had an extremely weak lytic effect on the mem-

branes. This was originally interpreted as a separation of active agents; however, assays of the supernatants showed that the relative strengths were comparable to those normally found in an extract. Thus one-eighth dilutions of extracts prepared by freezing and thawing resulted in membrane-lytic strengths equivalent to that of the above supernatants and cement dissolving titers from 4 to 8 which are also equivalent to that of the supernatants.

Dissolution of the membranes and intercellular cement is brought about by sperm extracts prepared by acidification or alkalization. A portion of a concentrated sperm suspension was brought to pH 1.5 by addition of HCl, centrifuged and the supernatant neutralized. The supernatant had nearly the same activity (Table 1) as a comparable extract prepared by freezing and thawing. Considerably weaker extracts were obtained by alkalization (pH 10 or above); however, the relative strengths of the lytic activities were the same as by other methods of extraction.

Sperm suspensions after heating to 100° C. for a few minutes yielded supernatants which dissolved the intercellular cement, but lacked the membrane lysin; however, this was not a differential extraction, since, as will be pointed out later, the membrane lysin is destroyed by heat.

It became evident that the lytic activities of sperm extracts were very closely associated and could not be separated readily by these methods into two specific fractions. Accordingly, further tests were carried out to determine some of the chemical properties of the active agents in the hope that these tests might lead to conclusions regarding their relationship.

The active agents of the sperm extracts are evidently large molecules as shown by their non-dialyzability. In a typical experiment 50 cc. of sperm extract were dialyzed in a cellophane tube for two days in an equal volume of sea water. Tests of the dialysate on the egg membranes and early cleavages were negative, whereas the solution inside the tube retained nearly the original strength.

Precipitates obtained by 50 per cent to 90 per cent saturation with $(NH_4)_2SO_4$ or by dialysis against distilled water contain the active factors. Precipitation by $(NH_4)_2SO_4$ was brought about by slowly adding at 3° C. a saturated solution of the salt to the sperm extract until the desired concentration was reached. At 50 per cent saturation a floccular white precipitate usually formed within a few hours. Since centrifuging tended to disperse rather than to concentrate the precipitate, it was allowed to settle out for a period of 24 hours and the supernatant decanted. This did not give complete separation, but was sufficient to show that the bulk of the activity of the sperm extract was in the precipitate. Table 2 records assays of the lytic activities of the precipitate and supernatant. The residual activity of the supernatant was probably due to incomplete separation of the precipitate and perhaps also to incomplete precipitation of the active substances.

A heavy and complete precipitation of the active factors was obtained at 90 per cent saturation with $(NH_4)_2SO_4$ (Table 2). Concentrations intermediate between 50 per cent and 90 per cent gave results similar to a 50 per cent saturation. No differential precipitation of active agents occurred.

Dialysis against distilled water also brings about precipitation of the active substances from a sperm extract. In a typical experiment, 50 cc. of sperm extract were dialyzed against 4 liters of distilled water at 3° C. Dialysis was carried out for 48 hours although a precipitate began to appear within several hours. Assays

TABLE 2

Precipitation of lytic activities of a sperm extract

Treatment of sperm extract	Time for dissolution of egg membranes		Titer of cement lysin
50% saturation with $(NH_4)_2SO_4$	supernatant	15 minutes	4
	precipitate	1/2 minutes	64
90% saturation with $(NH_4)_2SO_4$	supernatant	No activity	No activity
	precipitate	2 minutes	Very strong (titer not determined)
Dialyzed against distilled water	supernatant	15 minutes	4
	precipitate	1 minute	32
Precipitation by adding alcohol		No activity	8
Control sperm extract (prepared by freezing and thawing)		1 minute	64

of these showed high lytic activities of the precipitate as compared to the supernatant (Table 2).

Since a differential precipitation of the lytic factors did not occur, it cannot be concluded on the basis of the above results that there are two different lysins present in the sperm extracts. The presence of two active agents is, however, suggested by a differential effect of heat on the sperm extracts. Table 3 records the results of typical heat tests on a *M. edulis* sperm extract. Samples of the extract were heated at various temperatures for five minute periods and then assayed for the lytic activities. Five minutes at 60° C. was sufficient to inactivate partially the membrane lysin, but had little effect on the cement lysin. Nearly complete inactivation of the membrane lysin occurred within 5 minutes at 80° C.; however, sperm extracts after one hour at 100° C. still caused dissolution of the intercellular cement, but showed no membrane lysis.

TABLE 3

Differential inactivation of lytic activities of a sperm extract by heat

Treatment of sperm extract	Time for dissolution of egg membranes	Titer of cement lysin
5 minutes at 60° C.	5 minutes	64
5 minutes at 80° C.	20 minutes	32
5 minutes at 100° C.	No activity	16 to 32
1 hour at 100° C.	No activity	8
Control	2 minutes	64

The heat stability of the cement dissolving activity of sperm extracts depends in part on the pH of the extract. At pH 3 to pH 8 there is little difference in the rate of inactivation by heat; however, at pH 10 the cement lysin is completely inactivated within a few minutes at 100° C.

Alcoholic precipitation of sperm extracts causes complete inactivation of the membrane lysin and partial reduction of the cement dissolving activity. Chilled ethyl alcohol was slowly added to a sperm extract at 2° C. until a final concentration of 80 per cent was reached. A white precipitate formed which was freed of alcohol and dissolved in sea water. This solution exhibited no membrane-lytic activity; however, its cement dissolving titer was 4 to 8 as compared to 64 for the original extract.

The lytic activities of sperm extracts are destroyed by trypsin. In a typical experiment a trypsin solution was added to a strong sperm extract resulting in a final concentration of 0.1 per cent of the enzyme. After several hours treatment of the extract with trypsin, the extract had no discernible effect on the egg membrane or intercellular cement, indicating that the active agents had been destroyed.

Extracts, prepared by freezing and grinding, of various tissues (other than the testes) of adult mussels exhibited no lytic activities on the eggs.

Sperm extracts may be dried under vacuum without loss of activity, a procedure which has proven useful for obtaining concentrated solutions of the active factors. Concentration of sperm extracts by this method or by precipitation in salt free solutions led to the discovery of an inhibitor of the cement lysin. Although only a low percentage of eggs cleaved in a concentrated extract, the blastomeres of eggs that did cleave were nearly as cohesive as the controls. Assays of the extract showed a high titer of the cement lysin, but its effect was not noticeable until about a ¼ to ⅛ dilution of the original extract. Apparently there was an inhibitor in the extract which over a threshold concentration prevented the dissolution of the cement. Little is known about the nature of this inhibitor except that it does not inhibit the membrane lysin, and that it is destroyed in a few seconds at 100° C.

Species Specificity

Tyler (1939) reported that the membrane lysin from *Megathura* sperm does not dissolve *Haliotis* egg membranes and von Medem (1942, 1945) concluded on the basis of more extensive tests that the membrane lysins of various mollusks are to some extent species specific.

Active sperm extracts are obtainable from both *M. edulis* and *M. californianus* and cross tests were carried out to determine the specificity of the lytic activities. *M. californianus* extracts were able to dissolve the membranes and intercellular cement of *M. edulis* eggs; however, the reverse tests were negative. An *M. edulis* extract had a very weak lytic effect on the *M. californianus* egg membrane and no discernible effect on the intercellular cement.

A few cross tests were carried out on the eggs of other animals. *M. californianus* and *M. edulis* membrane lysins did not dissolve egg membranes of *Acmaea scabra, Mya arenaria, Urechis caupo,* and *Strongylocentrotus purpuratus* nor did sperm extracts of these animals have any effect on their respective egg membranes or the *M. edulis* egg membrane. With the exception of a few experiments, only the membrane-lytic action of the sperm extracts was tested since the membranes of most of the eggs could not be removed for conclusive tests of possible cement lysins. *M. californianus* sperm extracts, however, had no effect on the hyaline layer of sea-urchin eggs after removal of the fertilization membranes.

Discussion

For the purpose of comparison, the main characteristics of the lytic activities of *M. edulis* sperm extracts are summarized in Table 4. It is evident from inspection of this table that the characteristics of these agents closely parallel one another. Similarities exist not only in occurrence and methods of extraction, but also in the chemical behavior. There is no conclusive proof that there are two active agents

present in the sperm extracts although this possibility is strongly suggested by a differential inactivation of the activities by heat. The membrane lysin is extremely heat labile, being partially inactivated in a few minutes at 60° C. and completely inactivated in a short time at higher temperatures. In comparison the cement dissolving activities is relatively heat stable and is only partially inactivated after one hour at 100° C. This greater stability of the cement lysin is also reflected by retention of activity after precipitation in alcohol, whereas the membrane lysin is completely destroyed by this treatment.

TABLE 4

Characteristics of lytic activities of M. edulis sperm extracts

Membrane lysin	Cement lysin
1. Maximum activity obtained by acidification or freezing of sperm suspensions	1. Maximum activity obtained by acidification or freezing of sperm suspensions
2. Not obtainable from other tissues.	2. Not obtainable from other tissues
3. Non-dialyzable	3. Non-dialyzable
4. Inactivated by trypsin	4. Inactivated by trypsin
5. Heat labile	5. Relatively heat stable
6. Precipitated by dialysis against distilled water	6. Precipitated by dialysis against distilled water
7. Inactivated by precipitation in alcohol	7. Partially inactivated by precipitation in alcohol
8. Precipitated in 50% to 90% solutions of $(NH_4)_2SO_4$	8. Precipitated in 50% to 90% solutions of $(NH_4)_2SO_4$
9. Not inhibited in a concentrated sperm extract	9. Inhibited in a concentrated sperm extract

Precipitation by alcohol, $(NH_4)_2SO_4$, and salt free solutions strongly suggests that the active substances are proteins, which, by definition, would be globulins. Inactivation of both the membrane lysin and the cement lysin by trypsin also suggests their protein nature. The enzymatic nature and probable protein structure of the membrane lysin of *Megathura* sperm has been previously pointed out by Tyler (1939) and evidently the membrane lysin of *Mytilus* is similar in nature.

The mechanism of action of the cement lysin is not known. Although it is only slowly inactivated by heat an enzymatic action cannot be completely ruled out. It may act in a way so as to prevent coupling of calcium with the cement protein; however, it is unlikely that this is brought about by binding of free calcium in the sea water. Excess calcium in a sperm extract does not decrease the effectiveness of the extract in causing dissolution of the cement. Also if the mechanism were a binding of free calcium one would not expect a species specificity of sperm extracts. That a species specificity does exist is shown by the fact that *M. edulis* extracts have no effect on *M. californianus* eggs and furthermore they do not dissolve the hyaline layer of membraneless sea-urchin eggs.

Fauré-Fremiet and Thaureaux (1949) have described in detail the action of a number of synthetic detergents on the eggs of *Toredo norvegia*. Separation of the first blastomeres of *Toredo* occurs in weak solutions of detergents in much the same way as a sperm extract separates those of *Mytilus*. Supposedly, detergents are able to interact with and disperse proteins and it may be that the cement dissolving action of a *Mytilus* sperm extract has a similar mechanism of action.

It is unlikely that there is any relation between the cement dissolving activity of *Mytilus* sperm extracts and hyaluronidase of mammalian sperm. The latter is enzymatic in action and acts on the hyaluronate substance of mammalian tissue (reviewed by Meyer, 1947), but has no effect on the intercellular cement of blood capillaries (Chambers and Zweifach, 1947). Also the hyaluronidase-like substance of sea-urchin sperm (Ruffo and Monroy, 1947) has no effect on the intercellular cement of the sea-urchin embryo. It might also be pointed out that beef testis hyaluronidase has no perceptible effect on the membranes and cement of *M. edulis* eggs.

Runnström, et al. (1945, 1946), described a lytic activity of methanol extracts of sea-urchin sperm which apparently caused liquefaction of the egg surface as indicated, in part, by constriction of eggs into fragments at high centrifugal forces. The active agent is heat stable, dialyzable through cellophane, and is not inactivated by trypsin. A few preliminary tests of the cement lysin of *Mytilus* indicated that it did not alter the irregular shapes of unfertilized eggs or facilitate constriction of eggs during severe centrifugation. Thus the egg-surface lysin of sea-urchin sperm and the cement lysin of *Mytilus* show considerable differences in their chemical nature and effects on the eggs.

The function of the several lysins obtainable from the sperm of animals is not clear although possible roles in fertilization have been suggested. A reasonable explanation for the presence of the membrane lysin, as suggested by Tyler (1939), is to aid penetration of the sperm through the egg membrane to the surface of the egg. Although the agent causing dissolution of the intercellular cement may occur in physiological concentrations, it cannot be concluded without further work that it has a role in fertilization.

Summary

Extracts of *Mytilus* sperm contain a lytic substance, or substances, which causes dissolution of the egg membrane and the intercellular cement which binds the blastomeres together. Bioassays were utilized to investigate the occurrence and relationship of the active agents and some of their chemical properties. The lytic effects are exhibited by concentrated sperm suspensions and their supernatants obtained by centrifugation. Extracts prepared by freezing and thawing and by acidification or alkalization exhibit the same relative strengths of lytic activities.

The active agents are large molecules, as indicated by their non-dialyzability through cellophane. They are precipitated by ammonium sulfate, by dialysis against distilled water, and by alcohol. These properties indicate a protein nature of the lytic substances, an assumption which is further supported by the fact that they are inactivated by trypsin.

While there is no conclusive evidence that there are two separately acting lysins, this is strongly suggested, in part, by a differential inactivation of sperm activities by heat. The membrane lysin is heat labile whereas the cement-lytic activity is relatively heat stable. It is concluded that the latter effect is not brought about by a binding of free calcium of the sea water.

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CARTESIAN DIVER RESPIROMETER

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In this paper is described a modification of Linderstrøm-Lang's Cartesian diver method (1937) specifically adapted for the measurement of slight variations in the respiration of cellular objects. The diver is a further development of the diver used by Zeuthen (1946) for measuring respiratory rhythms in the egg of the frog.

Essential Features of the Method

The diver (Fig. 1) has a gas volume of only about 1 μl. It is kept floating at a constant level throughout the experiment. Therefore no disturbing currents are set up in the flotation medium by the diver itself and the saturation equilibria within the diver (see below) are not influenced by such more or less sudden changes in pressure in the gas bubble as would result if the diver was allowed to go to the bottom of the flotation vessel between the measurements. The gas volume in the diver is confined to one air bubble only. Therefore only one meniscus separates the gas space in the diver from the surrounding medium. All these factors are considered essential for obtaining accurate respiration measurements. One more factor is of paramount importance: since the diver is supposed to float in a medium which does not itself flow, it is necessary to avoid heat convection currents in the flotation vessel. Such currents will result from temperature variations in the thermostat. Not only the magnitude of possible temperature variations is important, but also the rate of variation should be kept low. A very fine thermostat, operating with as gentle heating and cooling as possible, is necessary. If not, the temperature regulation itself, usually operating on the principle of alternating heating and cooling, will result in convection currents in the flotation vessel. Therefore, in most cases it is much more convenient to work in a well-stirred water bath which is not regulated at all, but which adjusts itself to the room temperature. If the water bath is large and the room temperature fluctuates only slightly, the temperature will change very slowly. In this way no disturbing convection currents are initiated. The total change in temperature of the bath can be kept within very moderate limits if the room temperature is relatively constant. The change in pressure in the diver is automatically adjusted for temperature changes because the other side of the manometer is connected to the enclosed gas volume in a large bottle (2 liters).

The diver consists of a diver chamber, bottom droplet with animals, gas bubble, and alkaline, CO_2-absorbing neck fluid. The gas bubble forms a complete separation between bottom and neck fluids (this point is discussed at some length by Giese and Zeuthen (1949)). A hollow glass stopper is inserted in the neck of the diver. The stopper serves several different purposes: it retards diffusion of gases to or

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from the surroundings, gives the diver buoyancy and permits the pressure-sensitive air volume of the diver to be reduced almost ad libitum. Since the glass stopper is inserted without any grease, narrow passages are left which allow the air bubble in the diver to react with volume changes to outside pressure variations, created by the aid of an adjustable water manometer. The diver floats in the same medium which is in the neck of the diver; e.g., in alkaline water, isotonic with the biological medium in the bottom droplet. A long glass extension may be attached to the outer end of the stopper. This makes the diver float upside down ("inverted diver"), so that the organisms in the diver, if immobile, will come to rest on the meniscus between air and water, not on the bottom of the diver, away from the air bubble.

FIGURE 1. The diver.

Experiments with the diver are reported in the paper by Zeuthen (1950b). Figure 2 in that paper indicates that the diver is practically tight to gases. However, in the first part of the experiment, an apparent respiration is often measured. This is explained by assuming that a diffusion equilibrium is gradually becoming established inside the diver. If, for instance, the diver is filled with fluids which are in equilibrium with air, but an oxygen bubble is introduced, gas goes in solution because oxygen is more soluble in water than is air: Equilibrium is established asymptotically. How long it takes before it is well enough established depends mainly on the gases involved, on the relative amounts of water and gas in the system, and on the diffusion distances in the water of the diver. In the diver used for the experiments on eggs of *Urechis,* relatively much water was present in the system. Therefore the initial equilibration period was long (1–2 hours). It is important to remember that for disclosing a possible rhythm in respiration, the initial period need not be over. If it is not over, the rhythmic respiration is merely superimposed on a smooth curve which asymptotically approaches the X-axis.

During the respiration experiment, the pressure in the system decreases. At the same time as oxygen is used from the bubble, gas which is dissolved in the water in the diver again goes out of solution, back to the bubble. The condition of equilibrium spoken of becomes a quasi-equilibrium in which the oxygen tension in the water and in the bubble drops at the same rate. Thus oxygen is taken from water and from bubble in proportion to the amounts of oxygen present in both places. In the author's experiments on *Urechis,* approximately 6 to 10 per cent of the oxygen was present in the water, and the total respiration is measured too low by this value. For the measurement of variations in oxygen uptake this means practically nothing (comp. Linderstrøm-Lang, 1946). However, as soon as absolute respiration measurements are to be performed, these conditions have to be taken into consideration by applying corrections or by reducing the ratio between water and gas in the diver.

Any variation in respiratory rate must show up somewhat delayed because a diffusion path is introduced between the respiring objects and the air bubble. In the inverted diver (with objects on the meniscus) this is usually not a significant factor, however. In the experiment on *Urechis* (diffusion distance less than 0.5 mm) the delay was probably less than 1–2 minutes. Moreover, the fluctuations in respiration were recorded without any significant damping. These statements are theoretical deductions based on Linderstrøm-Lang's calculations (1946). However, the experiments of Blinks and Odenheimer (personal information), who used this diver in studies on photosynthesis in single algal cells, seem to verify these deductions. If the dimensions of the diver are changed from those shown in Figure 1, Linderstrøm-Lang's paper (1946) on diffusion systems in micro respirometers should be consulted.

Making, Calibrating and Filling the Diver

The diver is made from a thin-walled capillary tube with a diameter conveniently a little less than 1 mm. The ratio between inner and outer diameter should be close to 0.9. Such capillaries may be drawn from thin-walled test tubes which should be heated by rotation in a broad flame. From the thin-walled capillary two pieces are selected, of which one fits tightly into the other. The former piece is used for the stopper, the latter for the diver chamber. Principles of glass blowing which are given by Holter (1943) are employed. For the micro-burner a hypodermic needle is very convenient. With rich natural gas (as in California), the oblique tip of the needle serves to mix the gas and air. It is best to work with a very small flame (3–4 mm long) and it is often useful to mount the micro burner on the table of a dissection microscope. It is desirable to keep the volume of the sealed stopper small (3–4 times the volume of the "pressure sensitive" gas bubble) in order to make the diver as sensitive to pressure changes as possible.

Before use the diver should be tested and calibrated. For this purpose the diver chamber is filled completely with water and the stopper is inserted below water. Since the cross section of the capillary used for making the diver is usually not quite circular, the stopper will only slip into the diver chamber if it is correctly oriented. It requires some practice to find this orientation, and it may be useful to mark both tubes conveniently. This, however, has not been done by the author because very often useful reference marks (small dots, easily recognizable curvatures and the like) can be found on the diver. The diver thus filled should sink in its normal flotation

medium. If so, small air bubbles are introduced into the diver chamber until the diver floats. For the introducing of air bubbles the diver is taken out of the medium and the stopper is gently pulled back, without completely removing it, however. Small air bubbles will then enter the diver along the stopper. If the gas volume of the floating diver is too large or too small, glass is removed from or added to the tail of the stopper. The many small air bubbles in the diver are allowed to unite into one. Then, below water, the stopper is pulled out and the air bubble is taken into a calibrated Holter braking pipette (Holter, 1943). The amount of air to be introduced into this diver before each experiment is indicated by the length of the air column in the braking pipette used.

A great advantage of the present method is that the diver can be charged free-hand. Before the experiment the diver is rinsed by sucking first sulfuric acid and then water through the diver chamber by means of an ordinary pipette which is introduced into the submerged diver chamber. The measured volume of air is taken up into the braking pipette, and after this the biological object(s). In the filled pipette, therefore, we have in the lower end the respiring cells, and in the upper end the measured air volume. The pipette is gently blown out into the diver chamber which has previously been completely filled with water. If done under the dissection microscope it is fairly easy to deposit a large number of cells in the bulb of the diver and to place the air bubble where desired in the capillary, thus trapping the cells. After the experiment the cells may be removed from the diver and counted. It has (rather unexpectedly) been found that a complete and safe separation between bottom droplet and neck seal is formed if the air bubble is big enough to make a circular but very short (about 0.2–0.3 mm.) zone of adherence to the glass wall. Creeping of fluid along the glass from neck to bottom has only been observed when dust particles happened to form visible bridges across the clean glass surface. After the neck fluid has been replaced with isotonic alkali which is run in with a pipette (care being taken not to touch the air bubble), the stopper is introduced and the diver is transferred by pipette into the flotation vessel. The flotation vessel, manometer, microscope, and thermostat, have been described by Holter (1943); for thermostat, however, see above.

Measuring the Equilibrium Pressure

When the respiration of an object is to be measured over short periods the equilibrium pressure as well as the time must be measured very accurately. The following procedure of "turning point determination" has given good results: the diver floats at about the same level all the time. Just before a reading is to be taken, the manometer is adjusted at such a constant level that the diver rises very slowly. However, due to the respiration processes, the diver becomes gradually heavier, so that it rises more and more slowly, comes to a standstill, and finally starts sinking. It is possible to make the diver turn at exactly the same level ($\pm$ a few 0.01 mm.) in all measurements. When the diver turns, a stopwatch is started. The watch is stopped again when the next reading is due, and at the same time another stopwatch is started for measuring the next period, etc. In this way the pressure remains unchanged from just before to just after the diver turns, and the two branches of the manometer can therefore be read (to the nearest 0.1 mm.) immediately after the watches have been pressed. The equilibrium pressure measured is valid for the

moment when the diver turns and this moment can be observed with an accuracy of some few seconds. The described method of measuring equilibrium pressures works best when the equilibrium pressure changes fast.

Sensitivity of Method

The accuracy of the method was studied in connection with the experiments on dividing *Urechis* eggs. With a diver having an air volume of 1 μl. the oxygen consumption measured in 5-minute periods was about $2.5–5 \times 10^{-3}$ μl. (change in equilibrium pressure 2.5–5 cm./5 min.). This respiration could be measured with an error of probably well below 1 per cent. Thus the sensitivity of the method is about $2.5–5 \cdot 10^{-5}$ μl.

Summary

A Cartesian diver method which is specially adapted for following variations in respiratory rate of small objects is described. Accurate respiration measurements can be obtained every few minutes. The diver is calibrated empirically and it is filled free-hand.

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RESPIRATION DURING CELL DIVISION IN THE EGG OF THE SEA URCHIN PSAMMECHINUS MILIARIS

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Introduction

In a previous paper (Zeuthen, 1946) it was reported that during early development of the egg of the frog (*Rana plathyrrhina*) each cell division is accompanied by a slight increase in oxygen uptake. The curve describing oxygen uptake as a function of time roughly approximates a sine curve. The division furrow of the first, second, and third cleavages always appears during a period of increase in oxygen uptake.

The experiments were performed in modified Cartesian divers. The rate of decrease in pressure in an oxygen bubble situated close to the egg was measured. The respiratory carbon dioxide, however, was not chemically absorbed, but held in solution by the egg and by a considerable volume of water bathing the egg. The diver system, of course, was not shaken, so that all exchange of gases had to take place over certain diffusion distances.

Both factors mentioned, *viz.:* the incomplete separation of O_2 and of CO_2 in the diver and the diffusion distances present in the system, make it somewhat doubtful how to evaluate the results. Linderstrøm-Lang (1946) made elaborate calculations on the results obtained by Zeuthen and concluded that the observed waves are probably slightly delayed and somewhat damped as compared with the curves which would describe the true respiratory processes in the egg itself. According to Linderstrøm-Lang the wave amplitude found (about 5 per cent of the respiratory rate in the minima between the waves) would probably indicate "true waves in the egg" with a maximum amplitude of 7–11 per cent of the respiration in the minima.

These experiments do not quite conform with those of Brachet (1934, 1935) working with the Fenn-respirometer. He found two considerably larger variations in respiratory rate per mitosis in the egg of *Rana fusca*. The discrepancy between the present author's experiments and those of Brachet remains unexplained. Brachet has expressed the hope of being able to repeat his experiments (1945).

In a recent letter in *Nature*, Tang (1948) claims many years ago (Tang, 1937) to have found slight mitotic variations in oxygen uptake in the sea urchin egg, however only at a high temperature (25° C.). Tang considers that the failure of Gray (1924) to find any change in oxygen uptake in the dividing sea urchin egg is due to the fact that the experiments were performed at a low temperature (11° C.). Tang as well as Gray used the Warburg technique. Unfortunately, Tang has not been able to supply a copy of the original paper which was published

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in a Chinese journal. All records were destroyed during the war. Also Runnström (1934) found slight fluctuations in respiration in dividing sea urchin eggs. According to this author there is a maximum of respiration during prophase.

None of the experiments mentioned, however, have appeared convincing to the reviewers in the field. The problem of respiration during mitosis is not solved yet. Linderstrøm-Lang's calculations on Zeuthen's experiments on the frog egg indicated that in future experiments with the Cartesian diver we would have to employ more intensely respiring eggs than the frog's egg, and we would have to secure complete carbon dioxide absorption in the diver. Both conditions are fulfilled in diver experiments on eggs of the sea urchin *Psammechinus miliaris* to be reported in this paper (a short note on this work has already been published in *Nature*, 1947). The curve found for this egg must therefore be considered rather accurately to describe the true processes in the egg itself. The experiments on *Psammechinus* are concentrated on later divisions (nos. 4–8), and the main object of the investigation is to establish possible mitotic variations in the respiration of this egg. The experiments were carried out in July–Aug., 1946. In a following paper on the egg of *Urechis* the first three or four divisions are considered, and on this object the exact time relations between respiration and stage of mitosis are given.

Material and Methods

Eggs of *Psammechinus miliaris* (Z-form) were fertilized artificially. It was not always possible to obtain batches of eggs which divided 100 per cent and synchronously, but if those eggs which were the first to enter the first division were picked out with a braking pipette and kept separately, these eggs invariably were found to divide well (95–100 per cent) and synchronously (i.e., within 5 min.) through a series of successive divisions. For each experiment 300–400 eggs were isolated in this way, the jelly was removed with a pipette, and about 200 eggs were transferred to the diver; the rest were kept as controls outside the diver in the same thermostat. In the diver the eggs were densely packed, but they were very close to a bubble of oxygen. The oxygen supply was calculated to be ample. Usually the first 5–6 divisions could be observed in the diver itself (not on all eggs, but on a representative number of eggs). In some cases, however, and especially for later divisions, I depended on observations of the controls in determining the cleavage times in the divers. In earlier stages, where comparisons could be made between division rates in-and-outside the divers, the rate of development was found to be uninfluenced by the stay in the diver.

The way of securing egg material which divided synchronously restricted this investigation to the later stages of development. The divisions nos. 4–8 were studied, these divisions taking the egg from the 8-cell to the 256-cell stage.

Two types of divers are used—neither of them very different from the one previously described (Zeuthen, 1950a). Both float with the stopper upwards, however, so that the eggs lie on the bottom of the diver, separated from the air space by a thin layer of water. The distance from the most remote eggs to the air space did not exceed 0.7 mm. in any experiment. The diver which is shown in Figure 1-A is the most sensitive because it contains a very small oxygen bubble (about 0.2 μl.). This air bubble is not large enough to separate the neck fluid from the bottom fluid.

The fluid which fills the neck and bathes the eggs is a CO_2 binding buffer: glycylglycine m/10 or m/50 in sea water, with pH adjusted to 8.2 by the addition of NaOH (pK of the amino group in glycylglycine being 8.07 according to Schmidt (1938, p. 613)). The buffer solution is made isotonic with sea water. During the division periods with which we are concerned (Fig. 2), the m/50 buffer was not observed to influence the development of the eggs at all, but in the m/10 buffer a slight retardation of the later cleavages (7–8) was noticed, and the blastulae did not swell quite normally. In the diver the oxygen bubble was kept down, close to the eggs, by the aid of a tiny glass extension on the stopper. The diver shown has the exact dimensions of the diver used in the experiment reproduced in Figure 2.

The diver shown in Figure 1-B is almost identical with the one used for the experiments with *Urechis*, the main difference being that it floats with the stopper upwards. Figure 1-B gives the exact dimensions of the diver which was used in

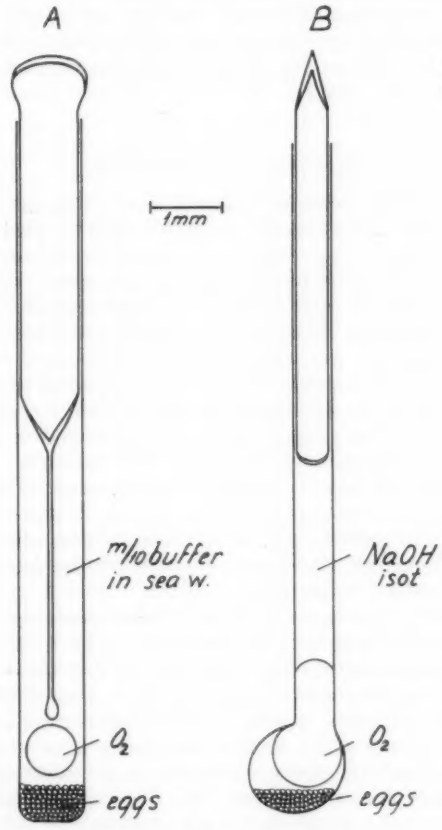

FIGURE 1. The divers used.

all three experiments of Figure 3. The air space (0.93 μl.) separates the bottom fluid with the eggs from the isotonic NaOH neck fluid. The diver floats in isotonic NaOH. Only the later cleavages (7–8) and the hatching period in *Psammechinus* have been studied with this diver. The accuracy of these experiments is not so high as it is now obtained in experiments with *Urechis*. Probably the temperature control in the thermostat (running sea water) could have been better.

Both types of divers were filled with pure oxygen. During the first part of the experiment, O_2 is used for saturating the fluids inside the diver. As mentioned elsewhere (Zeuthen, 1950a, c), this tends to make the measurements too high in the first part of the experiment. A possible respiratory rhythm should manifest itself even during this early period, however.

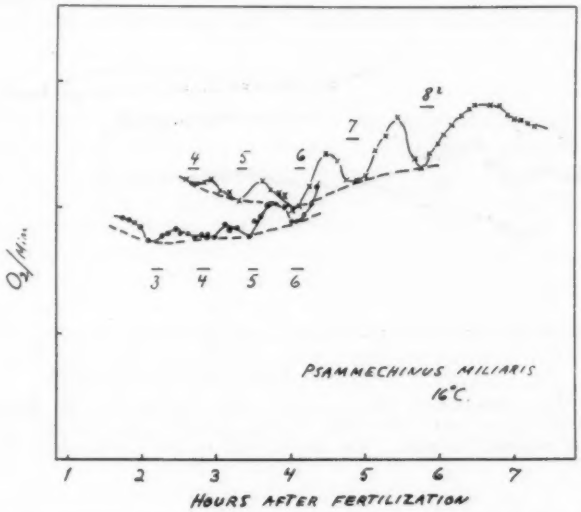

FIGURE 2. Two experiments with the diver shown in Figure 1-A, buffer m/10 glycylglycine.

The CO_2 absorption in both groups of experiments is considered complete. Since, moreover, the distance between the most remote eggs and the air bubble is small in all experiments, the damping of the respiratory waves found may amount to 5 per cent only, and the delay with which they are measured to less than two minutes. This can be determined from the tables in the paper of Linderstrøm-Lang (1946). Both the damping and the delay mentioned are insignificant, considering the not too high accuracy with which the dimensions of the respiratory waves can be recorded.

The temperature of the bath was regulated at about 16° by rapidly running sea water. The equilibrium pressure was determined as described by Zeuthen, 1946, 1950a (turning point determinations). The apparatus used was built in a very simple way at the Zoological Station, as far as possible following the directions of Holter (1943).

Experiments

Figure 2 shows two experiments with the diver of Figure 1-A. The ordinate gives the respiratory rate in terms of change (in cm. Brodie) per minute of the equilibrium pressure of the diver. The abscissa gives the time in hours after fertilization at 16° C., the 5th cleavage occurring 3 hours, 20 minutes after fertilization.[2] Each measurement is plotted in the middle of the period for which it is valid.

After the initial period with too high readings is over, the true slope of the two curves is indicated. The main trend of the curves seems to conform fairly well with the S-shaped curve of Lindahl (1936) showing the increase of respiration of

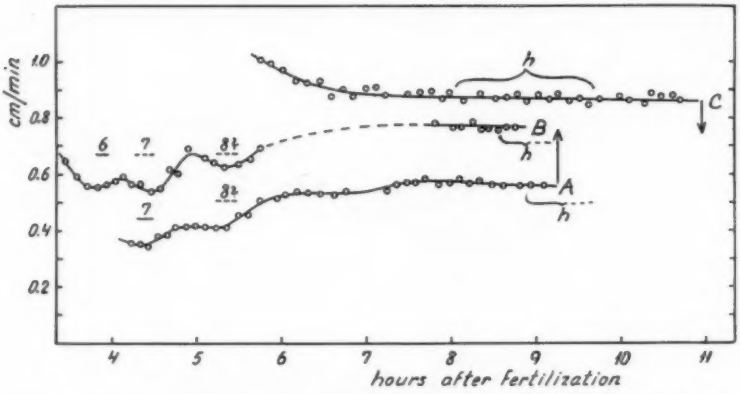

Figure 3. Three experiments with the diver shown in Figure 1-B. Actually all three curves should have been plotted at the same level as curve B. For the sake of clearness the curves have been separated in space. The arrows indicate the displacement. *h* indicates the hatching period.

the sea urchin egg during the cleavage period. However, waves are superimposed on this curve, one wave per mitosis. The amplitude of the waves increases as development proceeds and as more and more simultaneous divisions occur in the embryos. The cytoplasm cleaves during the period of decreasing respiration, in some cases perhaps even when the respiration is at a minimum.

All experiments of Figure 3 were performed with the diver shown in Figure 1-B. These experiments differ from those of Figure 2 in that the eggs lay in

[2] Dr. Hans Borei, Stockholm, has pointed out to me that the time relations on my curve in *Nature* are not quite correct (Borei, 1948). In *Psammechinus* the 5th cleavage (at 16° C.) appears 3 hours, 20 min. after fertilization, not after 4 hours, 30 min., as indicated in my figure. I have not myself measured the time (at 16° C.) from fertilization to start of the single experiment, because the necessary manipulations during this period involved serious temperature changes. From this and other reasons the said curve was only intended to give a rough survey to indicate for the reader on which part of the respiratory curve in the sea urchin the respiratory waves are located. However, I thank Dr. Borei very much for his information which I have taken advantage of in timing the curves of Figures 2 and 3 of this paper in relation to the time of fertilization.

natural sea water throughtout the experiment, CO_2 being chemically absorbed by the alkaline neck fluid. The curves of this figure form a continuation of those of Figure 2. The eggs were introduced into the divers at the 64-cell stage or even later, and in one experiment (C) the respiration was followed until hatching of all eggs had taken place in the diver. Saturation equilibria are not established until about 1 hour after start of experiment. Cleavages 6 to 8 seem to be accompanied by large variations in respiratory rate; later the respiratory variations again become slower and slighter, and for a long time after, no variations whatsoever in respiratory activity were disclosed, despite the fact that all the larvae hatched one by one during this period.

The general trend of Figure 3: a continued rise in metabolic rate followed by a period of constant respiration during the period of hatching conforms again with Lindahl's S-shaped curve—in this case with the later half of the S.

Discussion

The experiments indicate rather conclusively that the mitoses in the sea urchin egg are accompanied by changes in respiratory rate. But the interpretation remains uncertain in this respect: Is the increase or the decrease in respiration the important thing? As a matter of convenience, we shall in this paper interpret the curves as indicating that each mitosis is accompanied by a slight excess oxygen uptake. The respiration increases at some early stage of the mitosis, attains a maximum and decreases again at the end of the mitosis when the cytoplasm cleaves.

A smooth auxiliary line, which just touches all minima of the experimentally found curve, will separate a number of small areas above the auxiliary line from a large area below this line. According to the accepted point of view the large area below the line would indicate some kind of "basal metabolism" of the eggs, whereas the small areas would measure the "excess metabolism" of the individual mitoses.

The upper curve of Figure 2 shall now be analyzed in detail in order that we can get some idea of the relative magnitude of the "basal" and of the "excess" metabolism of the division cycle numbers 5–8 incl. in the egg of *Psammechinus*. There is a difficulty here because the measured respiration is known to be too high in the initial period of the experiment (see above). In this particular experiment, however, the diver had been in the flotation vessel for about ¾ of an hour before measurements were taken. Calculation by the formulae of Linderstrøm-Lang (1946) indicate that after about another 1.5 hour the initial period of this particular diver (dimensions fig. 1-A) is practically over. The curve found seems to agree well with this view. After the 6th division the general trend of the curve conforms well with the curve of Lindahl. In the upper curve of Figure 2 we may therefore introduce a reasonable correction for the measured respiration in the first part of the experiment by extrapolating the curve found after the 6th division back to the time of 5th and 6th division. In this way we find that the respiration was approximately 25 per cent too high during the 5th division and about 10 per cent too high during the 6th division. This way of correcting is admittedly rather arbitrary, but in view of the comparatively low accuracy of the whole analysis, it is probably satisfactory.

Table 1 gives the results of the analysis.

TABLE 1

Division number	Number of cells dividing per embryo	Wave area	
		Absolute	Percentage
5	16	1.0	3.4
6	32	2.1	5.0
7	64	3.9	7.7
8	128	5.7	9.6

The different areas between curve and auxiliary line touching all the valleys are determined by weighing the areas after cutting the graph paper. In Table 1, column 3, the extra O_2 (absolute measure, independent on correction) taken up during the 5th division is taken as unity and it appears that during the 8th division approximately 6 times as much extra oxygen is consumed as that taken up during the 5th division. As indicated in column 4 the "excess oxygen consumption" is but a small fraction of the basal oxygen consumption in the same period, the figures being 3.4 per cent for the 5th division and 3 times as much (9.6 per cent) for the 8th division. The reason why the percentage extra O_2 increases less than 3-fold at the same time as the absolute extra O_2 increases 6 times is partly due to the fact that the "basal" metabolic rate increases with development, but mostly because also the time occupied by one division increases. It is unknown whether this longer division time in later stages is due to an extension of one or more stages of the mitosis proper, or to the appearance of an interphase. Although only one experiment has been considered good enough to permit a close analysis, qualitatively this experiment is supported by the other experiments published. The two experiments of Figure 2 are in good agreement in indicating the approximate size of the respiratory waves accompanying divisions 4–6. Also, the important experiments of Figure 3 show that the large waves accompanying the later divisions can be found when no artificial buffer systems are added to the sea water. In these experiments the waves were found in the initial period, e.g. before saturation equilibria had become established in the diver. For this reason we shall not try to make a quantitative comparison with the experiments of Figure 2.

The safest and at the same time the most promising conclusion to be drawn on the basis of Table 1 is that for the three successive divisions 5–6–7 there is a doubling of the "excess oxygen consumption" for each new division. Thus, over this period the extra oxygen taken up per mitotic cycle is approximately proportional to the number of cells dividing. This finding is very different from what was found for the first three divisions in *Urechis* (Zeuthen, 1950c) and in the frog (Zeuthen, 1946). In these two eggs the extra oxygen taken up per mitotic cycle is essentially constant despite the fact that the number of dividing cells increases from 1 to 4. In the early divisions the *Psammechinus* egg (comp. the lower curve in Fig. 2) seems to line up with two other eggs mentioned, so probably it is safe to conclude that in dividing eggs some physiological difference exists between the early and the later mitoses.

Table 1 seems to indicate that after the 7th division in the *Psammechinus* egg matters change again. The extra oxygen consumption of the 8th division is only 46 per cent higher than that of the 7th division. Figure 3, curve B, however, shows an 8th respiratory wave which is approximately twice the preceding one. In this experiment no buffer was added. Both figures (Figs. 2–3) agree in indicating a big 9th wave which is very extended in time, though. After this no more respiratory fluctuations could be detected although ciliation and hatching of the embryos took place in the diver. It is well known that the mitotic rhythm characteristic for the dividing egg does not continue forever. The data of Table 1 and the curves of Figures 2 and 3 are perhaps best explained by the assumption that at some time—presumably around the 7th–9th division—the mitoses in the *Psammechinus* egg gradually become significantly asynchronous, less numerous and perhaps restricted to specific areas of the embryo. We are gradually approaching the period of differentiation with which this paper is not concerned.

For the deep interest in the problems involved and for valuable help during the performance of the work at Kristinebergs Zoologiska Station the author is very much indebted to Professor John Runnström, University of Stockholm.

Summary

The later divisions in the egg of *Psammechinus miliaris* are accompanied by variations in oxygen uptake. As in the embryo more and more cells divide simultaneously, the respiratory variations become more and more pronounced. The phenomenon is interpreted as an extra O_2-uptake correlated with the mitosis. During the four divisions nos. 5–8 proportionality is approximated between number of cells dividing and extra O_2 taken up. Thus, for the division period considered, the extra O_2 consumed by a dividing cell approximates constancy, *viz.*: it is independent of the size of the dividing blastomere.

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RESPIRATION AND CELL DIVISION IN THE EGG OF URECHIS CAUPO

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In a preceding paper (Zeuthen, 1950b) respiratory variations have been shown to accompany the later divisions in the egg of the sea urchin *Psammechinus miliaris*. In the frog egg the first three divisions are accompanied by faint, but clear-cut variations in oxygen uptake (Zeuthen, 1946). However, special conditions pertaining to large eggs in general (cf. the discussion in this paper) make it necessary to perform studies on a smaller egg similar to those on the frog egg. The present paper deals with the respiration of dividing eggs of the echiurid worm *Urechis caupo*. It is attempted to obtain accurate information about the shape of the respiration curve and to establish the time relations between respiration and stage of mitosis. Moreover, the recent communication by Tang (1948), claiming that in the sea urchin mitotic variations in respiration can be found at high but not at low temperature, has induced the present author to perform experiments on *Urechis* at widely different temperatures.

Material

The eggs of *Urechis caupo* have proved to be excellent objects for embryological and cell-physiological studies. From male and from female animals uncontaminated sperm and eggs can be drawn throughout most of the year and each animal furnishes good sexual products over a long period. The descriptive embryology of *Urechis* has been studied extensively by Newby (1940). After the addition of sperm the eggs undergo reduction divisions. These are followed by regular mitotic cell divisions, one division every 45 minutes at 15° C. Fertilization is usually almost 100 per cent and the divisions are synchronous within a few minutes in the same lot. After the 16 cell stage has been reached, the spiral cleavage of the egg becomes apparent, i.e., within the single embryo different quarters of blastomeres divide at different rates. The present study, therefore, is confined to the period in which the reduction divisions and the first 4 regular mitotic divisions take place.

Methods

The Cartesian diver method as described by Zeuthen (1950a) was used. The diver had an air volume of 0.8 μl–1.1 μl. The water volume was 2–3 times this. Thus in the experiments with O_2 in the diver 90–94 per cent of the oxygen present was in the gas phase of the diver; hence 90–94 per cent of the oxygen consumed by the eggs was manometrically measurable.

[1] Fellow of the Rockefeller Foundation.

After fertilization, 10 minutes were allowed to pass in order that it could be ascertained that the eggs were fertilized (to nearly 100 per cent). Then the diver was charged and transferred to the flotation vessel. After this about one hour elapsed before saturation equilibrium was established in the diver and reliable measurements could be obtained. Usually the mitotic divisions had not started until after this period, so that the 3 to 4 first mitoses could be studied.

The appearance of the cleavage furrows could be observed in the diver itself through the horizontal measuring microscope. Not every egg in the diver could be observed, but a representative number could always be followed. After the experiment the eggs were removed from the diver and brought under a regular microscope. The development was always as good as in the control outside the diver. In the diver the eggs lay densely packed in a layer the thickness of which, however, never exceeded 0.5 mm. Except in some few experiments at low temperature, about 400–700 eggs were employed in each experiment. As will appear below, the oxygen supply in all experiments was ample.

In the graphs the respiratory rate (expressed in cm. Brodie/min.) is plotted against the time. Each measurement is plotted in the middle of the period for which it is valid. The lines above the individual curve indicate the division periods.

Experiments

Diver without eggs, with unfertilized eggs, and with fertilized eggs

If a diver is charged in the usual way, but without respiring objects in the water, we obtain curves of the types I or II in Figure 1. In experiment I the diver contains a bubble of atmospheric air, in experiment II it contains pure oxygen. In the first part of the experiment we measure an apparent respiration, which is faint in experiment I, but very pronounced in experiment II. In both cases the apparent respiration can be explained by assuming that a saturation equilibrium is becoming established in the diver. If the diver is charged with an air bubble, there is a good chance that the fluids of the diver from the beginning are in equilibrium with the gas phase. The fact, however, that the diver will usually have become a little heated by the hand during the filling procedure may explain why, even in this case, we often measure a slight uptake of gas during the first part of the experiment (decrease in solubility of gases with increasing temperature). In experiment II (O_2 in diver) the fluids of the diver were from the beginning in an approximate equilibrium with atmospheric air. In contact with oxygen, gas is taken up because oxygen is more soluble in water than is air.

Curve III shows the results with about 700 unfertilized eggs in the diver (O_2 in diver). After the initial period of equilibration within the diver is over, we measure a respiration which is gradually increasing, the increase, however, following a very smooth curve without any indications of a rhythm. Evidently, the accuracy with which the respiration over short periods can be measured is very high, the largest deviations from the average being about 1 per cent and the error on the single measurement being very much smaller.

Curve IV shows an experiment with about 500 fertilized eggs in the diver (O_2 in diver). All eggs divided almost synchronously, and simultaneously with the eggs outside the diver. The lines numbered 1–3 above the curve indicate the division

periods as they could be observed in the diver itself. The time interval indicated by each line shows the whole division period. The bulk of the eggs, of course, divided within a distinctly narrower time interval. The respiration of the eggs fluctuates in waves: there is one respiratory wave per cell division and the cleavage period of each mitosis appears just after the respiration has passed the maximum.

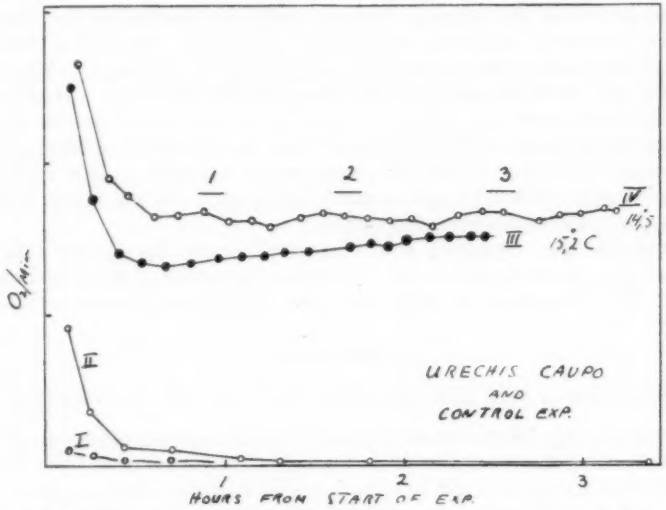

FIGURE 1. Control experiments with empty diver (I, II), experiment on unfertilized eggs (III) and on fertilized eggs (IV). 1st, 2nd, 3rd cleavages are indicated with horizontal lines.

A comparison between curves III and IV leaves little doubt that the respiratory waves are significant. Moreover, they have been found in all, e.g., about 10 experiments.

Experiments with dividing eggs at different temperatures. Oxygen in the diver

Tang (1948) considers the size of the respiratory waves accompanying cell division in the sea urchin egg to be highly influenced by temperature. The eggs of *Urechis* divide at temperatures ranging from about 9° C. to about 24° C. Above and below these temperatures cleavage becomes abnormal or fails altogether so that syncytia may develop. Experiments were carried out with normally developing eggs at 9.8–10° C., at 13–15° C. and at 22.8° C. Some of these experiments are assembled in Figure 2. The units on the ordinates have in each case been chosen so that the curves are directly comparable. The lower the temperature, the slower is the development and the less intense the respiration. Therefore, it is technically difficult to demonstrate the respiratory rhythm at low temperatures. The waves, however, are present at all temperatures. We shall return to this point in the discussion.

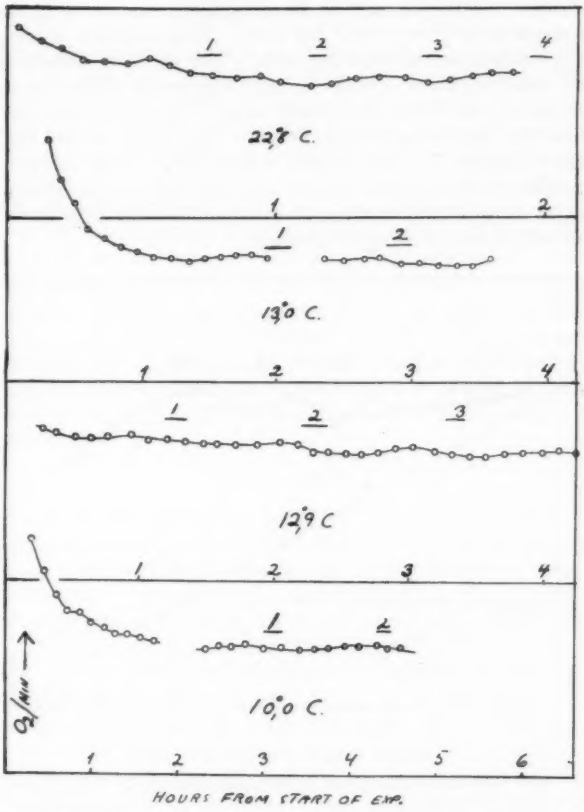

FIGURE 2. Within wide limits the respiratory waves appear to be uninfluenced by temperature.

Dividing eggs in a diver containing atmospheric air

The eggs seem to develop just as well in the diver where a high oxygen tension is prevailing as they do in the control (an open Petri-dish floating in the same thermostat as is used for the diver experiments). However, the respiratory waves might conceivably be influenced by the high oxygen tension in the diver without the cell division itself being in any way affected. The experiment, the result of which is shown in Figure 3, however, would seem to rule this possibility out. In this case the diver is filled with atmospheric air, and the eggs respire and divide normally until the 4th cleavage is completed. After this time signs of oxygen deficiency appear: respiration goes down and the cells divide more slowly than the controls outside the diver. The oxygen tension (expressed in per cent of one atm.) can, with fair accuracy, be calculated from the change in equilibrium pressure of the diver, assuming the oxygen pressure to be 20.9 per cent of an atm. at the moment when the diver is closed.

Curve II indicates oxygen pressure in the air bubble calculated in this way. The critical value seems to be about 6 to 7 per cent. This of course corresponds to even lower oxygen pressures on the respiring cells. The pressure difference between the gas phase and water which will allow enough oxygen to diffuse to the respiratory centers in this particular diver is evidently small. In most cases the divers which contained an oxygen bubble had about the same dimensions as had the diver used in the experiment of Figure 3. This would indicate that, in the oxygen experiments, the oxygen pressure in the eggs themselves was very close to one atmosphere. Thus, mitotic waves in oxygen consumption of dividing *Urechis* eggs have been demonstrated within wide limits of oxygen pressure.

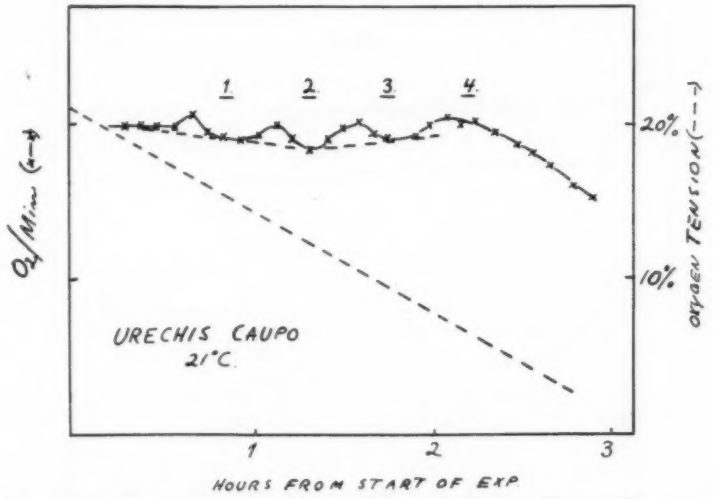

FIGURE 3. The respiratory waves can be demonstrated also at low oxygen pressures.

It may be worth while here to point out that the apparent difference between the curves of Figure 1 and Figure 2 is due only to a slight difference in the way of plotting. In Figure 1 the time scale is relatively long, and the respiratory waves therefore relatively long and flat. In Figure 2 the time scale is short and the waves shorter and steeper.

Oxygen uptake during the reduction divisions

There are some indications that the reduction divisions also result in slight changes in respiratory rate in the *Urechis* egg. In some cases the experiment was started so early that the reduction divisions occurred in the diver, though during the early part of the experiment at a time when the saturation equilibria were not yet established. However, two small waves seem to be superimposed on the curve, one wave per reduction division. Admittedly, these waves (to be seen on Fig. 2, 13°–10.0° C.) are very uncertain. Probably the safest conclusion to be drawn from these experiments is that, if the reduction divisions are accompanied by changes in the respiratory metabolisms, these changes are only slight.

Cytological observations

The time relations between stage of mitosis, cytoplasmic cleavage and fluctuations in respiration intensity were established in special experiments. Eggs from the same fertilization batch were kept in the diver and outside the diver at the same temperature and air or oxygen was bubbled through the outside suspension. Samples were drawn at regular intervals for cytological examination (Feulgen staining). The respiration was found to be low or increasing when the nuclei are in prophase. In those stages of actual mitosis when the nuclear membrane is dissolved away (meta-, ana-, and early telophase) the respiration is slightly increased. Maximum of respiration would seem to occur around metaphase-anaphase. Cytoplasmic division takes place during telophase at a time when respiration is already decreasing.[2]

The experiments indicate that the time relations between cytoplasmic cleavage and the different stages of mitosis are the same whether oxygen or atmospheric air is blown through the egg suspension. It should also be recalled that in all previous experiments the eggs cleaved at the same time in the diver (with air or with oxygen) as in the control (with air). All observations, tied together, support the general concept outlined above.

Discussion

The respiratory rhythm found in dividing *Urechis* eggs very much resembles that previously described for the frog egg (Zeuthen, 1946). Due to a series of complicating conditions, however, Linderstrøm-Lang (1946) considered that the observed respiratory waves in the frog egg, while qualitatively true, were slightly delayed and somewhat damped as compared with the events in the cells themselves. The chief difficulty was that CO_2 was not chemically absorbed but only held in solution by a large volume of water. Moreover, the diffusion distances between the air bubble and the metabolic centers in the large egg were too long.

In the present experiments advantage has been taken of Linderstrøm-Lang's calculations. In the inverted diver the respiratory CO_2 is chemically absorbed and the eggs lie on the water-air meniscus, so that the distance between the gas bubble and the most remote eggs never exceeds 0.5 mm. (exception: experiment at 9.8–10° C., see below). The damping factor, φ, of Linderstrøm-Lang is between 0.99 and 1.00, meaning that the damping of the respiratory waves is less than 1 per cent. The delay, t_D, is about 1 minute. Thus damping and delay are well within the experimental error. Changes in respiratory rate that are not too fast are picked up and recorded accurately with the instrument used.

The situation is slightly different with the experiment at 9.8–10° C. Because of the low respiration more than 1000 eggs had to be used. Therefore, the layer of eggs plus water separating the most remote eggs from the gas phase was 0.85–1.00 mm. The damping of the amplitude of the respiratory waves may in these experiments have amounted to 10 per cent, and the delay in recording the waves may have been 3–4 minutes. In view of the fact, however, that the waves are very long at this temperature, the delay is insignificant. In Table 1, a 10 per cent correction for the lower wave amplitude in these particular experiments has been applied. A comparison of the curves in Figure 2 reveals that temperature can hardly be con-

[2] The author wishes to record his sincere thanks to Dr. Irving A. Tittler, who made the microscopic preparations and studied the slides.

sidered significantly to influence the respiratory waves in the *Urechis* egg. If Tang (1948), however, considers that the respiratory waves are technically more difficult to demonstrate at a lower than at a higher temperature, we agree completely.

In Table 1 an intercomparison of all published curves is given. The wave heights are defined as described for the frog (Zeuthen, 1946). No certain dependency between the height of the respiratory waves and temperature or oxygen pressure can be observed. Figure 3 furnishes very slight evidence that the waves accompanying the later divisions may be slightly larger than those accompanying the first divisions. Figures 1 and 2, however, do not support such a view. The extra oxygen consumption of the first 3–4 divisions constitutes a very small fraction of the total respiration, e.g., 2–3 per cent only. Thus, the extra oxygen consumed per

TABLE I

	Wave heights in % of total					
	in oxygen					in air
Division no. \ Temperature	9.8°	10.0°	12.9°	13.0°	22.8°	21.0°
1	5.0	3.5	3.5	5.0	6.0	5.5
2	—	3.5	6.0	5.0	4.0	6.0
3	—	—	6.5	—	5.0	8.0

mitotic cycle is low, and probably it increases only slightly or not at all as we go from the 1st to the 3rd or 4th division. In this respect there seems to be a difference between the first divisions in *Urechis* and the later divisions (no. 5–8) in *Psammechinus*. It remains to be seen whether this is a difference between eggs or a difference between division stages, although the latter possibility appears to be the more likely one (Zeuthen, 1950b).

The physiological and biochemical function of the "extra oxygen" consumed during mitosis is unknown. However, since "mitotic variations" in oxygen uptake were found in a fertilized frog egg, which for some reason did not divide (Zeuthen, 1946), no close connection is indicated between respiratory processes and cytoplasmic cleavage. The fact that in *Urechis* (and *Psammechinus*) the cytoplasm always divides during a short period of decreasing respiration is an argument against the theories forwarded by some authors (discussed by Loeb, 1913) that the general increase in respiration which takes place while the sea urchin egg develops into a blastula correlates with the cytoplasmic cleavages and the resulting surface enlargement of the embryo. Moreover, comparing the eggs of the frog, the sea urchin and *Urechis* we find that the time relations between respiratory waves and cytoplasmic cleavage vary: In the two small eggs the cytoplasm cleaves in 2 or 3 minutes during telophase (decreasing respiration), but in the frog egg (*Rana fusca*, Brachet, 1934, 1935) the cytoplasm already starts to divide at the animal pole of the egg during prophase (increasing respiration). However, in the frog it takes a long time before the furrow embraces the whole egg. It seems probable that in all three eggs the time relations between stage of nuclear division and of respiratory changes may be

much the same as found in the egg of *Urechis*. Thus the bulk of evidence is in favor of the theory that cleavage is more or less dissociable from the two more closely correlated processes of nuclear division and fluctuations in oxygen uptake. The experiments of Runnström (1934) to be discussed below throw some light on this latter correlation.

According to Runnström (1934) the first part of the mitosis in the sea urchin egg is strictly dependent on the presence of oxygen. In an atmosphere of nitrogen or in the presence of KCN the eggs are not capable of carrying out those steps of the mitosis which lead to the dissolution of the nuclear membrane. If, however, the eggs are brought under anaerobic conditions or if they are poisoned with KCN at any stage after the anaphase (diaster), the eggs will divide synchronously with the controls in air. Unfortunately, such experiments have not been performed on *Urechis*. Provided both eggs are really comparable, it would seem that oxygen is required during the period of increasing respiration, but that it is not necessary when the respiration is again decreasing. Perhaps, therefore, an increase in respiration in the first part of the mitosis is ***indispensable*** for the mitotic process in the sea urchin and in *Urechis*. In the frog this is ***not*** so. Brachet (1935) found that this egg is capable of development to the young blastula stage in the complete absence of oxygen. Here fermentative energy evidently can take the place of oxidative energy even during the first part of the mitosis.

Zeuthen (1946) mentions the fact that in the frog egg the mitotic variations in O_2-uptake can be interpreted not only as variations in the rate of oxidations in the egg. Even if the egg respires at an absolutely uniform rate the rate of O_2-uptake from the surroundings will vary with possible changes in the permeability of the egg surface to O_2. In the latter case the "respiratory waves" measured will actually only be variations in the amount of oxygen physically dissolved in the egg. The presented accumulation of further experimental facts on other, more intensely respiring and probably more permeable eggs, however, makes a uniform interpretation on the basis of the permeability theory of all results almost impossible. There seems to be little doubt, then, that what we measure is in reality variations in the rate of oxygen utilization in the eggs.

My sincere thanks are due to Dr. L. R. Blinks and to Dr. C. B. van Niel, both of Hopkins Marine Station, for the interest they showed in the present work.

Summary

The first 3–4 mitotic divisions in the egg of the echiurid worm *Urechis caupo* were found to be accompanied by slight (2–3 per cent) increases in respiration. The phenomenon was found at all temperatures at which normal development takes place (9–24° C.), and it was found with air as well as with oxygen in the diver. The respiration was low or beginning to rise during prophase. It was maximum around metaphase-anaphase, lower during telophase when the cytoplasm divides.

In the discussion the experiments are considered in relation to previously recorded observations.

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COELOMIC PRESSURES IN SIPUNCULUS NUDUS

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Most invertebrate animals are characterized by lack of constancy of their internal pressure. In sipunculids, for example, the pressure may fluctuate within a short time between nearly zero and the equivalent of the blood-pressure of man. Pressure variations of this magnitude never occur within closed vascular systems. They are possible only in continuous spaces filled with fluid and enclosed by a muscular body wall. In such systems the main function of pressure is not circulation, but hydraulic work. In "closed" systems with more constant fluid pressure, hydraulic work is accessory. This latter function has been recently investigated in the annelid *Arenicola* by Chapman and Newell (1947). In the present study, pressure variations in *Phascolosoma* and *Sipunculus* will be analyzed mainly in relation to body movements. Coelomic pressure has long been recognized as essential in the digging activities of sipunculids; Uexküll (1903) measured in *Sipunculus nudus* pressures up to a maximum of 6 cm. Hg, i.e. 80.4 cm. of water, during burrowing in sand. Information on sipunculids is offered in the monograph of Baltzer (1934).

The hydraulic function of pressure in body fluids is wide-spread throughout the animal kingdom as shown in the following examples of water vascular systems. In holothurians pressure in the water vascular system is essential for tentacle erection (Zuckerkandl, 1948). In those sipunculids which possess tentacles, there is a water vascular system controlling their turgescence. In acanthocephalans, the water-vascular system is reported to be related to the erection of the proboscis (Hamann, 1891; Rauther, 1930).

In the polychaete *Arenicola,* burrowing depends on hydraulic pressure in the body cavity (Chapman and Newell, 1947). Pentastomids seem to be able to set up relatively high body fluid pressures, used for the extension of their parapodia and other appendages (Heymons, 1926). In priapulids, the evagination of both the proboscis and the caudal appendage is due to body fluid sent under pressure into the parts to be evaginated (Fischer, 1925). Bryozoa possess a hydraulic pump mechanism which serves the evagination of their tentacles (Delage and Herouard, 1897). In nemerteans the protrusion and reversion of the proboscis is brought about by coelomic fluid pressure, set up by the contraction of the muscular proboscis, while retraction of the proboscis is largely due to contraction of the retractor muscles (Böhmig, 1929). A similar mechanism of proboscis eversion and retraction exists in sipunculids (Baltzer, 1934).

In the foregoing examples, body cavity fluid or water vascular fluid is responsible for hydraulic action. Examples of hydraulic action of vascular blood are also reported. Polychaete and oligochaete annelids which dig channels in earth or sand (*Arenicolida, Glassoscolecida, Megaloscolecida, Lumbricida*) possess a strongly developed vessel running laterally along the oesophagus (vas extraoesophagale)

(Fuchs, 1906). Brücke (1925, p. 852) believes that turgescence of this vessel might favor digging. The leech *Herpobdella* has a capillary net on both its anterior and posterior suckers. When suction occurs, blood from the neighboring vessels streams into the capillaries, thus taking part in the mechanical effect (Emden, 1929). Also in *Herpobdella* the spermatophores are squeezed out during the sexual act by an increase of blood pressure in a specialized organ. In sexual function the hydrostatic pressure of blood is often used mechanically: e.g. for penis erection in mammals. In pelecypod molluscs the swelling of the foot is due to hydraulic action of the blood (Buddenbrock, 1939). The stretching of the ocular tentacle of the common snail is based simply on relaxation of the retractor muscle. The existing blood pressure is sufficient to extend the tentacle (Buddenbrock, 1939).

These examples suffice to show the widespread use made of body fluids for hydraulic work.

Materials and Methods

Phascolosoma gouldi was furnished through the kindness of the supply department at the Marine Biological Laboratory in Woods Hole, Mass. *Sipunculus nudus,* on which most of the work was done, was collected on the beach of Morgat, Brittany.

The *Phascolosoma* used were 6 to 10 cm. long, the *Sipunculus* sometimes reached a length of 18 cm. in the expanded state.

Pressure was recorded by inserting straight capillary glass tubes into the coelom of the sipunculid as used by Inada (1947) in crayfish and mussels. Capillaries with a bore of 1 to 1.5 mm. were selected and their zero point determined. The pressures are expressed in cm. of coelomic fluid. Temporary withdrawal of fluid from the body cavity into the capillary did not affect the animals. To obtain a coordinated record of pressures and body movements a method of registration on a kymograph was used. The moving meniscus in the capillary tube was closely followed by means of a horizontal metal thread which was connected through a lever system with a writing point, applied against the drum of the kymograph. While one person recorded pressures, another person inscribed on the kymograph record, by a system of conventions, the movements observed on the animal.

Discussion

Types of movement. In *Sipunculus,* three types of movement may be distinguished: swimming, defense movements, and burrowing.

Swimming is observed frequently in freshly collected specimens kept in sea water without sand. In the regular, beat-like bendings of the body all circular muscles are contracted, while the longitudinal muscles of the dorsal and ventral sides contract and relax alternately (Uexküll, 1903). Swimming movements are accompanied by alternate rises and falls in pressure.

When strongly excited, *Sipunculus* and *Phascolosoma* execute defense movements. The two extremities of the body may be brought together and often cross, while the body of the animal turns around its longitudinal axis. The pressure, during this action, is considerably higher than during swimming movements. In

Phascolosoma it reaches commonly 50 cm., in *Sipunculus* commonly 70 to 80 cm. of body fluid.

Usually the defense reaction does not consist in active movements, but in a state of immobility accompanied by high turgor and the reduction of the body surface. The longitudinal muscles contract to their maximum. In both *Sipunculus* and *Phascolosoma* pressures reach top values around 100 cm. of body fluid. There seems to be no relation between maximum pressure and body size. The highest pressure observed—108 cm.—was in a small specimen of *Phascolosoma.*

Burrowing type movements and corresponding pressures. On animals put in a glass jar filled with sea-water and devoid of sand, five to six phases may be distinguished in the cycle of burrowing type movements (*Sipunculus*):

1) At the start there is formation of a "hump," i.e., a bending upwards of the central part of the body in a nearly vertical plane. At the same time the pressure begins to rise from initial values of 2–3 cm. of water.

2) While the hump is maintained, the eversion of the proboscis begins speedily. The pressure continues to rise.

3) The proboscis is partly protracted (usually about two-thirds) while the hump is being released. From that instant on the progression of the proboscis is slower. Simultaneously the pressure, reaching a maximum, mostly between 10 and 30 cm., stops rising, and starts falling immediately. The fall is usually completed before the next phase begins.

4) The tentacles appear. They are set free by a slight recession of the anterior extremity of the proboscis. Pressure remains constant, or, sometimes, drops very little.

5) Tentacles and proboscis are being retracted. During this action, a further, very slight fall of pressure (2–4 mm.) takes place. This fall may fail to occur or set in only after completion of proboscis retraction. Pressure attains exactly or nearly the level at which it started at the beginning of the burrowing cycle.

6) A period of immobility, of constancy of pressure. This phase is usually skipped when the animal is in a state of activity. In that case, phase 1 succeeds phase 5.

When *Sipunculus* remains in its feeding posture, the cycle is interrupted after phase 4.

During fast burrowing, which is accompanied by no tentacular eversion, the pressure, in preparation for the next protraction, rises while the proboscis is being retracted. In that case, high pressure is already present when the retractor muscles suddenly relax. The proboscis is, as it were, shooting out against the sand in full force. Such cases are illustrated by Figures 1 and 2, drawn after kymograph records.

The formation of the hump is synchronized with rise in pressure and the disappearance of the hump with fall in pressure. On the other hand, rise and fall in pressure are not essentially related to the protraction and retraction of the proboscis, as might have been expected *a priori*. Indeed, hump and definite pressure rise may fail to occur, while the proboscis is protracted normally, although more slowly than under marked pressure (Fig. 3). In several instances, the pressure was seen to remain constant at 1 cm. of body fluid during the whole protraction-retraction

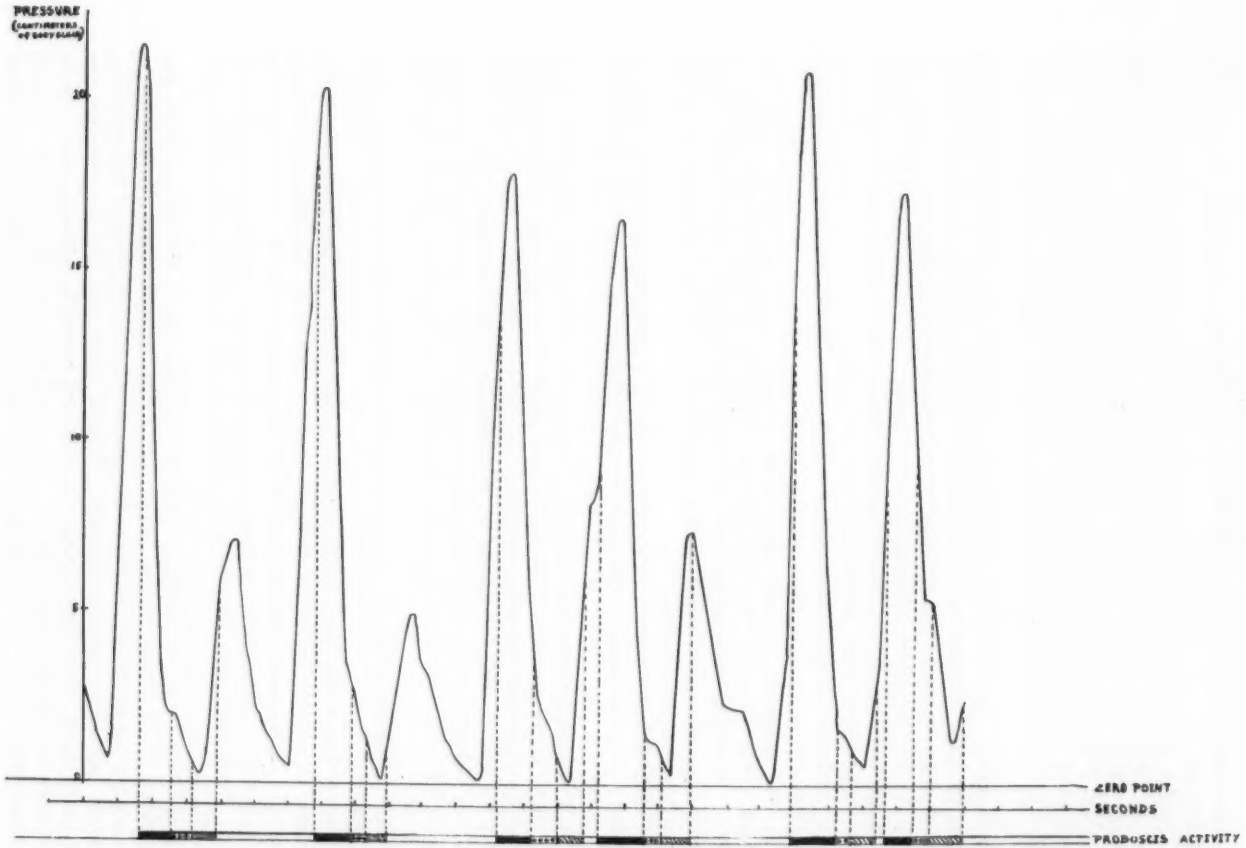

FIGURE 1. Data from kymographic record of coelomic fluid pressures in cm. coelomic fluid, and corresponding movements of proboscis of *Sipunculus nudus*. Time line: six second intervals. Proboscis activity indicated by following conventions: ■ proboscis being extended, ⊂⊃⊂⊃ proboscis extended, ▨ proboscis being retracted, ▭ proboscis retracted.

operation. However, protraction never occurred when the pressure was below 0.9 cm. of body fluid. The cessation of pressure rise and onset of fall may correspond to various states of protraction of the proboscis. Exceptionally the pressure rise may continue throughout the process of proboscis retraction. These irregularities illustrate the lack of strict coordination between pressure variations and proboscis protraction. Evidence of disjunction between the cycle of movements and the cycle of pressures is recorded by the kymograph record in Figure 1. There

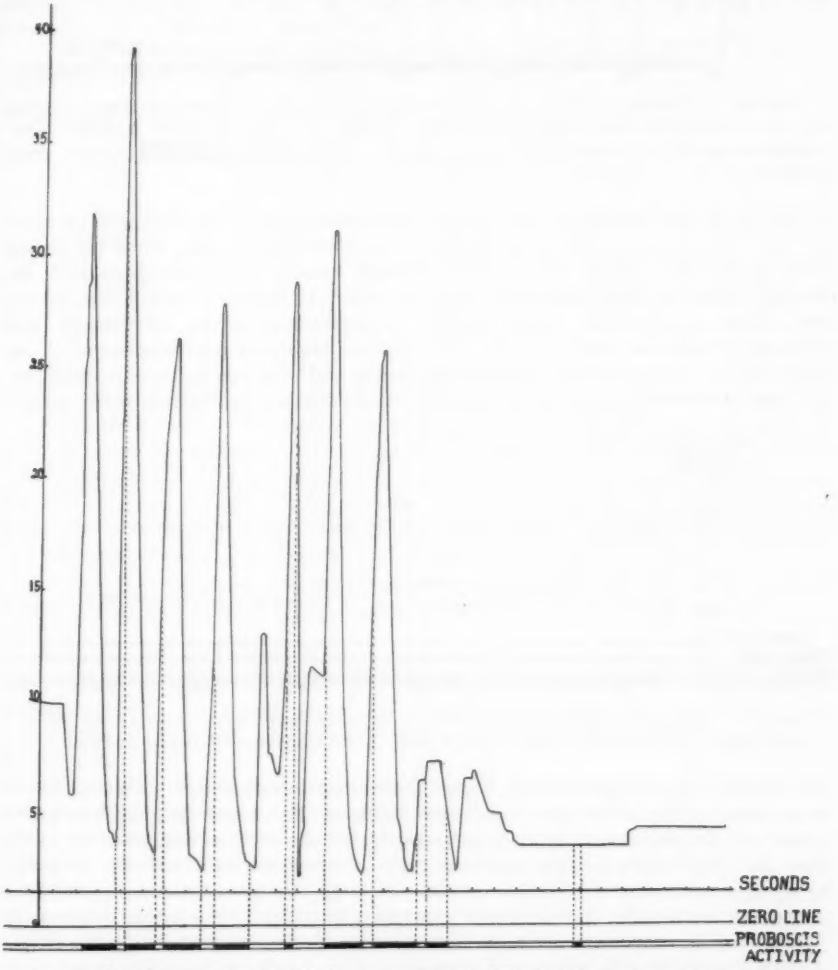

FIGURE 2. Pressure (ordinate) in mm. coelomic fluid in *Sipunculus nudus* recorded on kymograph. ▬ proboscis extending, ▭ all other phases of proboscis movements.

tended, ▭ proboscis extended, ▨ proboscis being retracted, ▭ proboscis retracted.

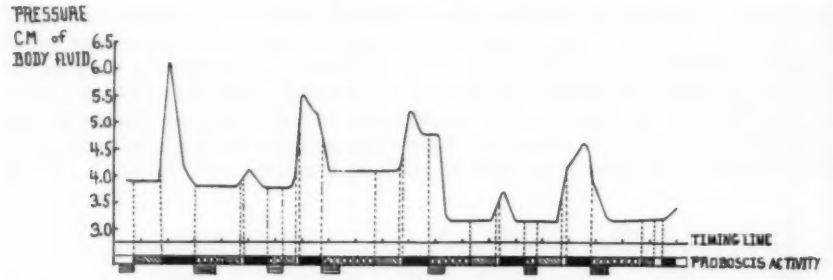

FIGURE 3. Pressure (ordinate) in coelomic fluid and activity in *Sipunculus nudus* recorded with aid of metronome beat every third second. Timing line gives 10 second intervals. Conventions for record of proboscis activity as in figure 1 with addition ▭ tentacles being extended.

we see, in several instances, one cycle of movements coinciding with two pressure cycles. The pressure cycle keeps firmly to its established timing, while the timing of the movement cycle is less constant; though keeping pace sometimes with the pressure cycle, at other times it is twice as slow. It should be noted that during what might be called an "extra-systole"—a pressure cycle not coordinated with proboscis protraction—the pressure never rises as high as it does otherwise. Even in this case a certain relation between movement and pressure is, then, maintained; very high pressures seem to be set up only when proboscis protraction really occurs.

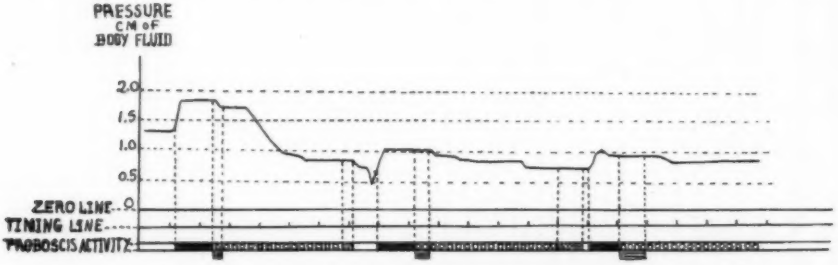

FIGURE 4. *Sipunculus nudus*, pressure and activity noted every fourth second with aid of metronome. Six second intervals on time line. Conventions for activity as in figure 3.

The 5th and 6th pressure rises in Figure 2 are illustrations of this. Here pressure has no time to drop to the usual minimum, before the rise accompanying protraction occurs and the movement cycle is not exactly half as slow as the pressure cycle. Thus the rhythmicity of the pressure cycle is upset to some extent. Depending on how soon after the "extra-systole" the large rise accompanied by proboscis protraction sets in, the "extra-systole" may be represented by a whole spike, as in Figure 1, or be reduced to a simple discontinuity of what appears to be one single pressure rise spike (6th spike on Figure 2).

The pressure rise may set in before the formation of the hump. In this case the appearance of the latter is characterized by a marked increase in speed of the rise.

The basic tonus, represented by the pressure minimum attained with phase 5, may remain constant during several consecutive burrowing cycles and then rise to a different level (Fig. 4).

The amplitude of pressure changes during burrowing movements in free water is very variable. In *Sipunculus* pressure during proboscis protraction was seen to attain maxima ranging from 1 cm. (Fig. 3) to about 40 cm. (e.g. animal I of table below). Maximum pressures registered during proboscis eversion in different specimens of *Phascolosoma* follow: 11.4; 12.4; 15.0; 16.0; 17.3; 21.3; 25.0; average: 16.9 cm.

The following table gives the relation between average pressure extremes and intervals between onsets of pressure rise in eight specimens of *Sipunculus*.

Animal	Average time elapsing between onset of two pressure rises (seconds)	Number of cycles averaged	Highest pressure reached (cm. of body fluid)	Average pressure minimum (cm. of body fluid)
I (Fig. 2)	12.7	8	39.6	2.6
II (Fig. 1)	15.6	11	21.2	0.4
III	17.3	6	20.1	2.5
IV (Fig. 4)	18	6	6.1	3.6
V	18	3	8.7	2.3
VI	18.4	6	25–30	—
VII	18.5	5	23.4	0.9
VIII	20.5	7	24.2	2.5

From these eight experiments, the average time elapsing between two pressure rises is about 17 seconds. The shortest time intervals are obtained in animal I which also showed the highest pressures, but in those with low maximum pressures the time intervals remain near the average (IV, V). The rhythmicity of pressures therefore seems largely independent of their magnitude. During most experiments the minimum pressures were nearly constant.

Proboscis eversion in *Sipunculus* occurs whatever the position of the animal, and not only, as Baltzer (1934) claims, when it lies on its ventral surface.

Pressures and movements during burrowing in sand. Specimens of *Sipunculus* were placed in a glass aquarium filled with sand, between a glass plate and the aquarium front. Since little space was available for lateral movements, the worm was often forced to slip along the aquarium front and its movements could thus be watched.

At the start of burrowing, pressures were similar to those given in the preceding section. As the animal gradually penetrated into the sand, the maximum pressures increased very considerably, the minimum pressures slightly. The increase in maximum pressures was sometimes strikingly gradual (Fig. 5). As long as only part of the animal's body was under sand, the maxima did not exceed those commonly observed during digging type movements in free water. Once the animal was about two inches below the surface, maximum pressures around 90 cm. were commonly reached (top value on record: 96 cm.). During these highest pressures greatest speed of progression was obtained. When the animal returned to low speed progression, the pressure maxima decreased markedly. Under com-

paratively low pressures the progression was never fast. High speed progression is not possible before the animal is wholly under sand. Indeed, the "hump" is effective under these conditions only as explained below. Since fast progression requires high pressure and is possible only when the animal is nearly completely under sand, pressure gradually increases as it becomes effective.

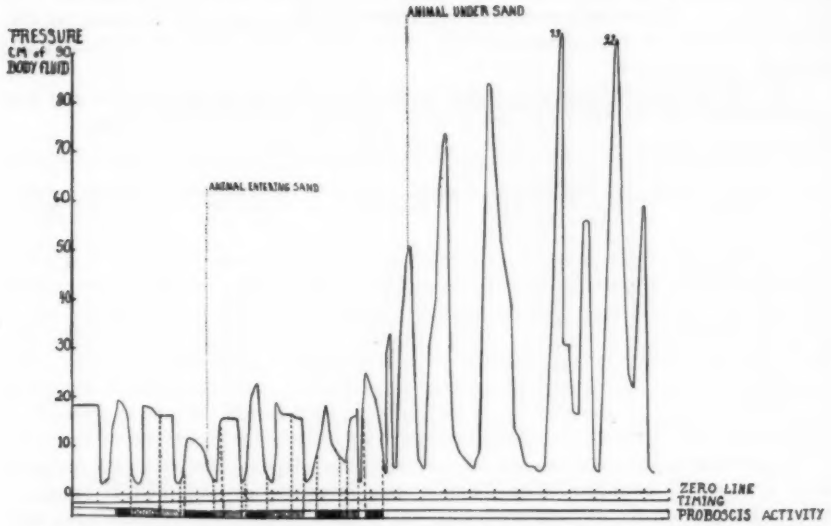

FIGURE 5. *Sipunculus nudus*, coelomic pressure and activity recorded every third second with aid of metronome. Conventions for proboscis activity as in figures 1 and 3. Time line, 10 second intervals.

Figure 6 shows how pressure variations and body movements are coordinated during burrowing under sand. The results are similar to those for burrowing movements in water except that the maxima often reach very much higher values. The period of low maxima corresponds to one of lower burrowing activity; the animal progresses less speedily and remains longer in the "feeding position" (proboscis and tentacles stretched out).

Under sand, proboscis protraction has been seen to continue under a pressure as low as 3.2 cm.

As in animals kept in free water, tentacle eversion was brought about by a retraction of the distal section of the proboscis. In this way the tentacles are set free within a cavity prepared beforehand by the burrowing proboscis and are protected from rough contacts with the sand.

Figure 5 affords evidence that the normal correspondence between burrowing cycles and pressure cycles may be systematically upset during burrowing in sand, just as it was occasionally during movements in free water. One burrowing cycle consistently corresponds to two pressure cycles. (The movements could not be followed during the later part of the record.) Proboscis retraction does not occur

around the time of a pressure minimum, as in the case shown by Figure 1, but coincides with the top of a pressure spike. Curiously, retraction occurring at the moment of a pressure maximum seems to prevent the pressure from falling. A pressure plateau is maintained near the maximum as long as retraction lasts. The pressure spikes are always influenced by changes in the proboscis cycle, yet the rate at which the spikes follow each other may not be. Thus the two cycles (pressure and proboscis movements) may occupy various positions in time with respect to each other.

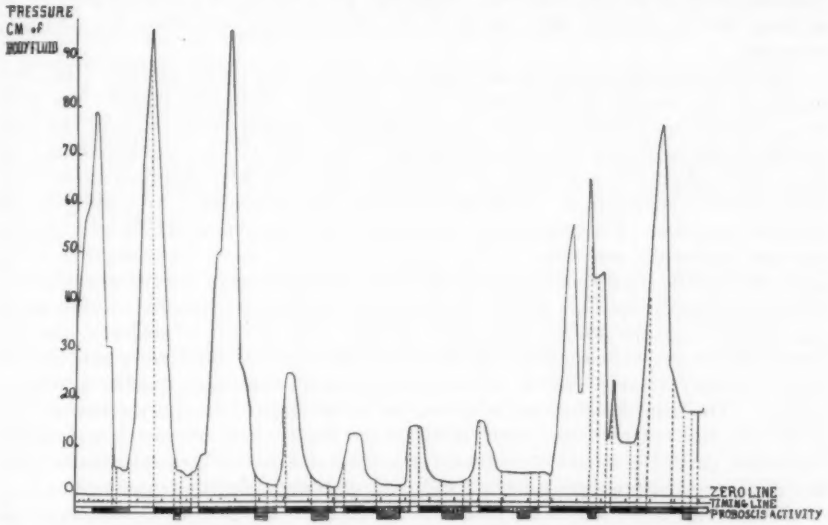

FIGURE 6. *Sipunculus nudus.* Coelomic pressures and proboscis movements recorded with aid of metronome every third second. Conventions as in figure 3. Time intervals, 10 seconds.

In conclusion, the motor nerve centers controlling contraction of the body wall appear to some degree independent of those for the movements of the proboscis, i.e., contraction and relaxation of the retractor muscles.[1] Moreover, pressure is likely to have a function partly independent of proboscis protraction.

The function of pressure during burrowing. With respect to its function, one may consider pressure from three points of view:

1) pressure necessary for proboscis eversion in absence of any resistance.
2) pressure necessary for the proboscis to overcome the resistance of the milieu.
3) pressure necessary to insure the progression of the animal in its milieu.

1. When the proboscis does not meet with any resistance, it can be protracted, as stated above, under exceedingly low pressure (minimum 0.9 cm.). Low pressure eversion differs from eversion under high pressure by being slow and very regular. A given low pressure is sometimes maintained although, with proboscis eversion, the

[1] The muscles of the proboscis wall are not important in protraction. See below.

volume of the body cavity gradually increases. Thus is brought out the ability of the relaxed *Sipunculus* muscle to compensate exactly for any variation in pressure by corresponding variations of its length, so as to maintain a constant state of tension.

In the absence of coelomic pressure, proboscis protraction was never observed. An active animal was cut open along the dorsal midline, so as not to injure its nerve cord. Thus deprived of the possibility of setting up pressures, it was put back into sea-water. Although the muscles of the animal remained able to display activity, no eversion of the proboscis occurred. Therefore the presence of a head of pressure of at least 0.9 cm. of body fluid seems to be the necessary condition for proboscis eversion.

The question was raised whether this condition is also a sufficient one. In other words, do the muscles of the proboscis play any active part in eversion? The body of a specimen of *Sipunculus* was cut through transversely at the level of the anus and the posterior part of the worm discarded. The anterior part was kept immersed for several minutes in a solution of iodo-acetic acid (M/50), supposed to interfere with muscular contraction. Then the proboscis was drawn back by pulling on the retractor muscles. The fragment of the animal was slipped over the tip of a wooden rod and kept under sea-water. The system was made nearly water-tight by tying a thread over the cut end of the worm stretched over the stick. A capillary tube was introduced behind the base of the proboscis for measuring pressure, and sea-water was injected into the body cavity with a syringe. At 1.1 cm. of coelomic pressure (measured in cm. of sea-water), the proboscis was seen to unroll very slowly, part way. As the pressure rose to 1.7 cm., the proboscis extended quickly and thoroughly. The tentacles came out at the end of its eversion. Since the muscles of the proboscis poisoned with iodo-acetic acid did not display any activity, it is probable that active participation of the proboscis muscles is not needed for proboscis eversion and that very low pressure is indeed sufficient to bring about this eversion.

What, then, is the role of the proboscis muscle? It seems to be at least two-fold. One function is the retention of the proboscis inside the body-cavity. The muscles of the proboscis seem indeed able to help the retractor muscles, if not to replace them in that role. In animals whose retractor muscles had all been cut, the proboscis still resisted pressure and did not unroll. A second role of the proboscis muscles must be related to the "thixotropic" effect of digging, which will be discussed below.

2. We shall deal now with the pressure necessary to overcome the resistance of the sand.

The experiment reported above (setting up measured pressure in the body cavity of an anterior portion of a *Sipunculus*, the proboscis muscle being kept from contracting) was repeated on the same fragment of *Sipunculus* under 2 cm. of sand. Pressure was raised up to 9 cm. of sea-water, while the proboscis protruded only very slightly and was unable to continue its eversion.

On the other hand, in a normal animal burrowing in sand, proboscis eversion was observed, in one instance, as reported above, under a pressure of only 3.2 cm. of body fluid, and in a number of ihstances under slightly higher pressures.

These two results seemed inconsistent. And *a priori* it would not have seemed reasonable to assume that pressures as low as 3.2 cm. could overcome the resistance of the sand. It was impossible to drive out by pressure, under sand, the inverted

finger of a rubber glove filled with water. The finger of a rubber glove surely meets a much higher resistance than the slender proboscis of *Sipunculus*. Nevertheless eversion of the proboscis in sand under a pressure of 3.2 cm. seemed hardly understandable.

The work of Chapman and Newell (1947) appears to offer the clue to this question. These authors, studying pressures in *Arenicola*, showed that very low pressures are sufficient for penetration in muddy sand. This kind of sand, which also is the medium of *Sipunculus*, behaves as a thixotropic colloidal system. "If the sand is prodded several times with a stick its resistance diminishes, and it remains in a semi-fluid state as long as the prodding continues." With an anesthetized proboscis or the water-filled finger of a rubber glove this thixotropic effect cannot be obtained, hence the failure, in these cases, to obtain protraction in sand under low pressure. However, the extending proboscis of *Sipunculus* probably has an effect similar to the prodding of a stick, and in this respect the muscles of the proboscis must play an outstanding role.

3. The function of high pressure, unnecessary to proboscis eversion even under sand, seems to be to insure efficient progression of the worm in the sand. Large pressure rises have been reported always to be accompanied by a bending of the central part of the body. This bending also occurs under sand, as far as compatible with the resistance of the walls of the burrow. The central part of the worm is thus immobilized on a given spot by the pressure exerted against these walls. The body is therefore prevented from being pushed back during the action of proboscis protraction. At the same time, the high pressure insures a speedy progression of the proboscis, thus increasing the "prodding" effect. High pressure seems therefore to have a triple role: to maintain the hump, which serves as anchorage in the burrow; to favor the thixotropic action of the proboscis; and to insure speedy progression.

One may wonder how the animal can do without the hump during the last slow phase of proboscis eversion. It should be remembered that the hump is not in all circumstances a necessary condition for proboscis progression. Indeed, when the proboscis first penetrates into the sand, the rest of the body, including the hump, is still outside it. Nevertheless the animal is able to progress slowly.

Yet during the more energetic phase of burrowing, the hump, no doubt, affords a necessary fulcrum. It corresponds to the "collar," a swelling appearing on the distal extremity of the proboscis. It is assumed that, due to a grip on the sand at this swelling, the body can be drawn forward.

The hump has another important function. It creates on one side a hollow space between the wall of the burrow and the animal's body. In this space a backward flow of sand particles was consistently observed. Apparently the sand stirred up by the proboscis during the first phase of its protraction is thus flowing back, leaving ahead the space necessary for the progression of the body. The body has indeed a much larger diameter than the proboscis and could not follow the proboscis, if the latter burrowed a channel of just its size. The effects on the sand of the thixotropic stirring action of the proboscis were thus directly observed, and in relation to them the hump appears to be indispensable. Considering the resistance of the walls of the burrow, this hump can develop in sand under high pressure only.

The necessity of high pressure for progression in sand was shown by Uexküll (1903). He destroyed in a specimen of *Sipunculus* the posterior part of the nerve cord. The posterior part of the body was distended, high pressure could not be developed and the animal was not able to progress in the sand. If Uexküll then cut off the paralyzed posterior section and closed the opening with a rubber stopper, the anterior half of the worm was seen to disappear in the sand within a few minutes.

The role of pressure in circulation of coelomic fluid. Circulation of coelomic fluid in *Sipunculus* is brought about by several factors: the ciliation of the coelomic epithelium (Mayer, 1929), the stirring action of the urns—little ciliated organelles swimming around freely in the body cavity—and the movements of the body wall. The effect of cilia is probably the only one acting consistently in the sense of fluid movement in a closed circle throughout the body.

It is clear that a mechanical action on body fluids cannot be qualified as being "circulatory" in nature, unless it shows some rhythmicity and constancy during at least a certain length of time. The question was raised whether digging, constituting one of the few rhythmical activities of the sipunculid, may have a sufficiently regular influence to cause circulation of coelomic fluid.

In order to study the effects of pressure variation during burrowing movements from this point of view, a few small air-bubbles were injected into three specimens of *Sipunculus*. The animals were placed on a glass plate over a light so that the bubbles were visible. Care was taken to keep the animals in horizontal position. A regular movement of air bubbles during proboscis movements was observed in the anterior region of the body only. At the start of proboscis eversion the bubbles were shifting anteriorly, at the start of retraction caudally. Few shifts were observed in the middle and posterior regions. It is concluded that during burrowing movements, significant pressure gradients are rhythmically established in the front region only. They have an effect on the movements of coelomic fluid.

Some simultaneous measurements of pressure in the anterior and posterior part of the body were compatible with the air bubble experiment, the pressures in the posterior capillary rising and falling faster than those recorded by the anterior capillary.

Summary

The relationship between coelomic pressures and body movements has been investigated in *Phascolosoma gouldi* and, mainly, *Sipunculus nudus*.

During "defense movements," pressure in *Phascolosoma* is commonly 50 cm. of water, in *Sipunculus* 70 to 80 cm. During "defense immobility," the highest pressure recorded, in *Phascolosoma*, was 108 cm. There does not seem to be a relation between body size and maximum pressure.

Six phases of burrowing movements have been distinguished in *Sipunculus* and related to coelomic pressure. An important pressure rise coincides with a bending movement of the central part of the body. This "hump" and the high pressures associated with it have functions which are described. Very low coelomic fluid pressures suffice to cause eversion of the proboscis. Pressure is a necessary and sufficient condition for proboscis eversion in free water, while in sand the proboscis muscles seem to play an essential role during proboscis eversion in relation to the thixotropic property of stirred sand.

During most experiments, minimum pressures were very constant, while maximum pressures varied a great deal. During burrowing movements in free water, the highest pressure recorded in *Phascolosoma* was 25.0 cm., in *Sipunculus* 39.6 cm. of body fluid. During burrowing in sand, pressure of 96 cm. water was reached by *Sipunculus*.

The frequency of pressure cycles seems to be independent of the absolute pressure.

The coordination between the cycles of pressure and proboscis movements is variable. Therefore the retractor muscles and the muscles of the body wall are probably dependent on nervous centers which are not closely associated.

Pressure variations during burrowing have an influence on circulation of coelomic fluid.

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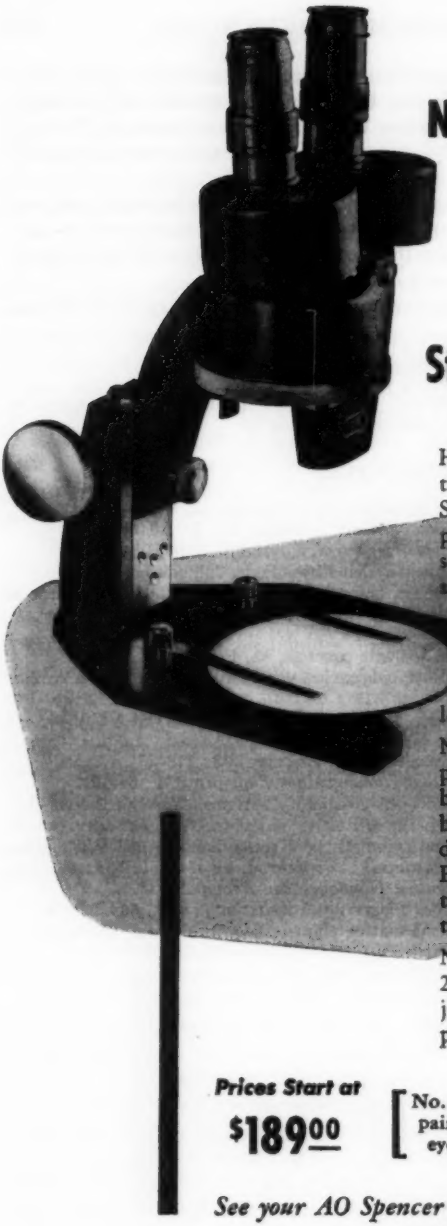

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